

How to stop the space station

Protests against the proposed space station Freedom continue, but the US Congress is likely to approve the project. The time has come for counter-arguments of a different kind.

LAST week, in an extraordinary show of unity, the presidents of 14 US scientific societies, representing both the physical and biological sciences, called on the US Senate to kill the controversial space station "Freedom" which, within recent weeks, has been voted down and then up again by the House of Representatives. The gist of the presidents' arguments was this: Freedom, at a conservatively estimated cost of \$30,000 million, cannot be justified as a valid scientific experiment. Worse, if spent, the \$30,000 million would be lost to "real" science even in cognate fields such as satellite communications and remote sensing research.

Last week's gang of 14 — representing the American Physical Society, the Geophysical Union and the Society of Zoologists, among others — is not, of course, the first to argue against the space station on the grounds that it has no substantial scientific purpose. But the gang's argument will not be listened to. For all practical purposes, the pro-Freedom lobby concedes that the space station cannot be justified as a remote, even a weightless, science laboratory. White House science adviser D. Allan Bromley is among those who say that science is not all; instead, he says the argument is between "investments in the future and current consumption".

Bromley's White House colleague, budget director Richard Darman, is even more lyrical: Freedom may not be the best possible route to space exploration, but where we would be now if Columbus had been forbidden to set sail or if the pioneers who settled the American West had stayed East waiting for the development of aircraft to convey them to California in greater comfort than the horse-drawn wagons in which they travelled? And there are less high-sounding arguments to be heard in favour of Freedom. US relations with Japan and Europe would suffer deservedly if the United States backed out now when those countries have contributed millions of dollars (see *Nature* 351, 428; 6 June 1991) — a worthy consideration that has not always carried weight in Washington. And then, as if Freedom were a hangover from the days of the Public Works Administration of the 1930s depression, people are busily calculating how many thousands will lose their jobs, or at least will not enjoy jobs that do not yet exist, if Freedom is not assembled.

At the heart of the issue is the fascination of the United States with exploration, or at least with adventure. The Apollo programme is still affectionately alive in many

people's memories (and often wins a mention in the community's defence of the research enterprise). However rational, the weakness of the argument that Freedom serves no useful scientific purpose is that it leads to a self-serving conclusion. It is as if the professional community is saying in killjoy fashion that, if the space station takes money from research, then the community is against it — whatever pleasure it might give the United States or even the world at large.

Earlier this week, the first Senate committee vote seemed to endorse the position taken by the House of Representatives; it seems likely that the full Senate will follow. What the gang of fourteen should learn from these developments, however unwelcome, is that the only effective counter to the arguments for Freedom is in coinage of the same kind. Of course, there are economic arguments, but they are not clinching; spread over ten years, Freedom will cost less than 2 per cent of the budget deficit if that persists at its present level.

So perhaps it is time to concentrate on guessing at and then telling what kind of an adventure Freedom will be. Mallory's view that people climb Everest "because it is there" is at least justified by the height of the mountain. And the Moon was "there" for Neil Armstrong, who reached its surface with aplomb. But what is to be made of an adventure to an orbit about the Earth in which people will simply go round and round in near-circles, doing as little as they can the while to disturb the solemn people busy on microgravity experiments and themselves at the beck and call of the logistics experts who will tell them what to eat, and when, and when the time has arrived to pack up and go home. There may be a little serious science to be done, but it will not be cutting-edge stuff. □

Bankrupt systems

The collapse of a British-based bank raises questions about the new world order as well as bankers' probity.

THE circumstances leading to the enforced shutdown last week of the Bank of Credit and Commerce International (BCCI) are not just a scandal, but a sign that we are a long way from what President George Bush has been calling the new world order. For what the collapse of this bank



has shown is that a sufficiently determined and ingenious group of people can make a monkey of regulations at the national level intended to prevent the theft of ordinary people's goods and chattels. The affair is especially embarrassing for Britain, where the bank was a bigish player and where no fewer than four unconnected trials of people accused of the dishonest manipulation of financial institutions are simultaneously under way in the courts.

Those in the research and other professions inclined to view these happenings as irrelevancies should reflect on three matters. First, the funds spirited away often belong in part to them, through their investments in pension funds and with insurance companies. Second, the scope of the irregularities now apparently commonplace in the financial world invite comparison with those which the research profession is from time to time preoccupied. Third, the prospects that the management of our global village will be informed by the civility of which individual nations are proud are forced, by these events, to recede.

Banks are rightly regarded as pillars of the establishment. More to the point, they are engines of economic and technical change. The principle, first recognized in Italy in the fourteenth century, is that they undertake to look after funds belonging to one class of customers (depositors) and then lend the same funds to a second class of customers (borrowers). When it works well, the banking system is a means of pooling the resources of many people to finance projects beyond the resources of individuals. How many of those who bought James Watt's first steam engines, for example, would have been able to buy them out of their own pockets? But when the banking system works improperly, as in the case of BCCI, it can be a way of pouring people's wealth into projects that cannot succeed (which betokens bad judgement) or, sometimes, simply into chosen people's pockets (which is fraud).

Almost from the outset, the dangers of malpractice have been recognized. Most jurisdictions require that nationally registered banks should keep in a form equivalent to cash (government securities, for example, or deposits with the central bank) at least a fixed proportion of their outstanding loans (laughably, in the light of recent experience, called "assets" in the banking trade). In some places, banks are forbidden to trade in the securities of public companies from fear that over-close involvement with necessarily risky businesses may put depositors' funds in hazard. (One of the cases now being tried in Britain involves an allegation that a subsidiary of an important bank improperly concealed the purchase of a public company's stock so as to manipulate its price.) The great collapse of the savings and loan industry in the United States, likely to cost US taxpayers \$500,000 million eventually, has different roots; the lifting in the early 1980s of restrictions on how the banks could use their funds while leaving the public guarantee of deposits (up to \$100,000) intact invited a host of bankers to make "heads I win; tails you lose" gambles.

Can anything be done? BCCI seems to have escaped some of the rigours of regulation by moving its registered office from London to Luxembourg, and to have plugged

some of the holes in its deteriorating balance sheet by persuading its principal shareholder, the ruler of Abu Dhabi, to put up extra funds. What proportion of the bank's supposed £23,000 million worth of assets is recoverable remains to be discovered, but there is a high chance that, yet again, more wealth will have vanished into thin air. It is by no means irrelevant that the total of the wealth spirited away in the past few years by the failure of banks and public companies is comparable with the sums of money required to solve major problems in the world — the burden of poor countries' debt, for example, and the cost of revitalizing the Soviet Union's economy (over which the heads of rich governments have been sucking their teeth this week in London).

The remedies are not tighter restrictions on what banks and other financial institutions are legally allowed to do, but closer and much more transparent scrutiny of what they get up to. The law requires that financial institutions should justify their conduct of affairs to their shareholders, but do they not also owe a similar duty to their depositors? And while it may be true that poor judgement by a banker is not the same as fraud, should not those whose judgement is consistently faulty be recognized as such more conspicuously than by the cancellation of their annual salary bonuses? □

Friends for France

The French government is trying out random (as distinct from anonymous) peer review.

THE French ministry of education has hit on a novel way of assessing the quality of those who teach at French universities. In the past few weeks, several people outside France have been sent letters by the ministry's director of research and graduate studies asking for the names of those French scientists whom the respondents consider to be "first-class scientists", of research groups "that would compare favourably" with others elsewhere and of people who have made "any contribution to science on a broad scale". Many recipients of the questionnaire have been nonplussed by it, not least because answers cannot be obtained simply by checking boxes.

There will, of course, be general sympathy for the ministry's ambitions. The French university system is growing quickly, as is the usual difficulty of telling who is good and who less good. It also makes sense that administrations should seek advice on such questions from outside their own parishes. (The German Max-Planck Gesellschaft has had great success with including scientists from overseas on its advisory councils.) But a general enquiry of the kind now put out is unlikely to provide the ministry of education with more than trouble. Some recipients of the circular will not answer, some will take endless quasi-judicial trouble and other will sing the praises of their chums. And what would happen if some French academics should set about soliciting responses of the third kind? □

Germany pulls back on space programmes

- European shuttle threatened by German cuts
- Space station module may also be trimmed

Munich

GERMAN support for a European space shuttle slipped last week to a new low, reducing the chances that the shuttle project, and the rest of the European manned space flight programme which depend on it, can be carried out as planned.

In his strongest statement yet against the French-led shuttle *Hermes*, German Research Minister Heinz Riesenhuber said on 11 July that he could no longer rule out a German withdrawal or a significant reduction in support for the shuttle.

Riesenhuber also said that cutbacks may be necessary in the space station module *Columbus*, the second of three large projects around which the European programme is built. (The third is the heavy launcher *Ariane 5*.) Germany is the main developer of *Columbus*, with a 38 per cent stake in the development costs.

A German withdrawal from *Hermes* could have far-reaching consequences for the European Space Agency (ESA) manned space flight programme as a whole. The renewed rumblings of a German withdrawal come just four months before a crucial meeting of the ESA council of ministers in Munich on 18–19 November to decide the future of the European programme.

A withdrawal at this late stage is possible because of a clause written into the original 1987 agreement on the ESA manned space flight programme. The clause states that any member state may withdraw from a project if the costs go over 120 per cent of the original estimates.

If Germany drops its support for *Hermes*, then Helmuth Dederra, head of space programmes at the German aerospace company MBB, predicts that an "avalanche" will follow. The French would probably withdraw their support for *Columbus* (with a 14 per cent stake, France is third behind Italy, which bears 25 per cent of the costs). ESA would effectively collapse, Dederra predicts, or at least require major restructuring.

The change of heart in Germany was brought on by a combination of budget cuts and cost overruns. The 1992 German federal budget left a shortfall of DM200 million (\$110 million) in the roughly DM300 million that Riesenhuber had requested for the three European projects. And Germany's long-range budget left a deficit of at least DM10,000 million for the three projects by the end of the

decade.

At the same time, the main contractor for *Hermes*, the French company *Aérospatiale*, two weeks ago submitted a bid of DM13,000 million that was more than 130 per cent of the original projected cost of DM9,500 million.

Officials in the German Research Ministry hinted that the Riesenhuber statements may have just been a negotiating ploy. "We do not really intend to drop both projects," said space policy official Gottfried Greger. Instead, Greger said, Riesenhuber will renew his efforts to persuade Germany's partners, especially France, to find a way to reduce the cost of the projects.

Greger added that Germany sees major problems with the *Hermes* project as it is now conceived. For one, it is doubtful whether *Hermes* currently has a large enough payload capacity to service *Columbus* and its free-flying component, the man-tended free flyer, properly. And if the payload capacity were increased, *Hermes* could no longer be lifted by the *Ariane 5* rocket unless the rocket itself were modified. Such modifications would cost additional money that ESA has not taken into account.

Furthermore, the cost overruns could be just beginning, said Greger. "It is bad news when a project is over budget right from the start."

One possible compromise, said Greger, would be to stretch out the *Hermes* project for two or three more years. Under this scenario, the first unmanned launch would take place in 2000 instead of 1998 and the first manned launch three years later.

But it is not clear if this would save enough money to satisfy Bonn, and this strategy has its limits. Greger declared — and Dederra of MBB concurred — that it would be "nonsense" to stretch the project for more than two years, as it will cost an estimated \$200 million extra for each year added and it would be impossible to hang onto the required technical and industrial expertise for a long enough time.

For its part, the French government played down the potential for conflict with Germany. Jean-Yves Le Gall, a special adviser to the French Minister for Equipment, Housing, Transport and Space, Paul Quilès, said that he "does not see a conflict" with Germany and that he is confident that the two countries "will arrive at a trade-off".

Le Gall said it was a normal occurrence in all countries that budgets are cut, but that nevertheless "projects go on".

The conflict between Germany and France over the European space programme has been bubbling beneath the surface ever since 1987, when Germany agreed to the projects only after much arm-twisting by the French. It has been primarily fear of a backlash in German-French relations that has kept Germany in line until now. But evidence of a new German attitude is clear in the words of Greger. If ESA and France do not come up with an acceptable plan by November, he said, "we will just pull out. No one can force us to stay in if we don't want to."

Steven Dickman

HIGH-ENERGY PHYSICS

Japan in retreat from SSC plans?

Tokyo

Is Japan going to turn down the United States' request for a substantial financial contribution to construction of the US Superconducting Super Collider (SSC)? When the matter was raised in discussions between Japan's Prime Minister Toshiki Kaifu and US President George Bush last week in Maine, Kaifu made no firm commitment one way or another, but a widely read Japanese daily newspaper says the Japanese government has already decided not to contribute funds.

In a front-page story on Friday, the Tokyo *Shimbun* newspaper, quoting an unnamed government source, reported that the Japanese government has decided not to contribute to the SSC because Japan needs to concentrate on rehabilitating its rundown national universities. Foreign Ministry officials last Friday (12 July) refused to comment on the article. But on Monday, following Kaifu's discussion of the matter with Bush on July 12, they said the article is "misleading".

At a press conference in Maine on 11 July Kaifu said: "There is growing awareness in Japan that this sort of thing, the Superconducting Collider, is important for science and technology, and researchers in Japan are studying what sort of cooperation would be possible. However, I am not prepared today here to say what sort of financial cooperation would be possible. And I might add that scientific and technological research in Japan is being carried under difficult financial situations as well".

A decision by Japan not to contribute would not be surprising. An official of Japan's Ministry of Education, Science and Culture, speaking on condition of anonymity last year, said he and his colleagues wanted to "run away" when approached by a US delegation from the Department of Energy for financial support in June 1990.

David Swinbanks

Cracks shut neutron reactor

London

DAMAGE to components in a French nuclear reactor facility last week forced officials to close the world's most powerful neutron source for repairs that are expected to keep the machine — and many of its 2,500 users — out of commission for two years.

Officials at the Institut Max von Laue-Paul Langevin in Grenoble first discovered cracks in a grid of the reactor, which is the heart of a thermal neutron generating facility, as part of a routine television-camera inspection in April, according to institute spokesman Brend Meyer. The damaged grid is part of a two-grid baffle that smoothes the flow of heavy water after it leaves the reactor. It seems that the lower of the two grids came loose and was pushed up into the higher grid. Officials suspect that radiation-induced brittleness in the grid material allowed it to crack under the strain.

"We first tried to readjust the lower grid. But when they tried to get the bolts out, they failed completely. Whether because of radiation or just corrosion, the bolts can't be loosened," Meyer said. Repairing or replacing the unit is likely to take at least two years, and even longer if the reactor must be drained of its heavy water.

The facility is used by more than 2,500 researchers, from biologists to material scientists, who visit the Grenoble facility for its powerful neutron beam. Scientists use the neutrons for basic research into neutron properties, as a 'microscope' to investigate the atomic structure of materials, and to examine viruses and biolo-

gical membranes at unparalleled magnification.

Although there are several other large neutron sources in Europe, none can match Grenoble's flux — 1.5×10^{15} neutrons $\text{cm}^{-2} \text{s}^{-1}$. Some users may be able to use the Saclay source in Paris, but a large new source at the Berlin HMI Institute that is closer to Grenoble's power is not yet running. Recently the European Communities, recognizing Europe's disproportionate reliance on Grenoble for neutron physics, gave the green light to upgrade a small Danish reactor at the Risloe research institute. But that is not yet completed, and the untimely failure of Grenoble will only reinforce the need for new facilities.

In the United Kingdom, the Rutherford-Appleton Laboratory's large neutron source would be a natural choice to absorb some of Grenoble's users, were it not for the fact that it is already oversubscribed by a factor of three. Government funding cuts threaten the facility's future, and laboratory officials are not eager to stretch their scarce resources on accommodating new users.

Late last week, officials from the three countries (the United Kingdom, France and Germany) that cooperatively run the Grenoble facility agreed — subject to approval by their governments — to use the shutdown as an opportunity to install a completely new reactor at the laboratory by 1994. Although it would be functionally identical to the existing reactor, a new reactor could extend the lifespan of the facility by over a decade.

Christopher Anderson

GERMAN SCIENCE

DFG selects new president

Constance, Germany

AT its annual meeting in Constance last week, the German grant agency Deutsche Forschungsgemeinschaft (DFG) selected Wolfgang Frühwald, a German language and literature scholar, as its new president.

Frühwald immediately announced that his priorities for expansion in the next few years will be biomedical research, molecular biology, and especially clinical medical research, where Germany, he said, has yet to become internationally competitive.

Frühwald will begin a three-year term as president in January 1992, succeeding ethologist Hubert Markl. Frühwald is the first DFG president since 1964 not to be a natural scientist. With legal scholar Hans Zacher as current president of the Max Planck Gesellschaft, two of the largest research organizations in Germany are now led by non-natural scientists.

On the future location of DFG, Frühwald says he thinks it likely that the organization will remain in Bonn even though the parliament and government are moving to Berlin. He supports the idea, currently in vogue in Bonn, to make Bonn a "science city", but he warns that this can be achieved only through a concerted effort by politicians and the scientific community, and not just by leaving a few scientific bureaucrats in Bonn after the move.

With an annual budget (in 1991) of DM1,300 million, DFG is the single largest provider in Germany of university research grants and also pays for thousands of stipends for doctoral and post-doctoral students. Supported by both the federal government and the *Länder*, DFG funds research in all fields, including both natural sciences and the humanities.

Steven Dickman

TELECOMMUNICATIONS

Mystery solved in US phone failures

Washington

THE cause of a string of major breakdowns in local phone service in several areas of the United States has been identified: a 'bug' in the switching program consisting of three or four faulty lines of computer code among the millions of lines that constitute the software.

Frank Perpiglia, vice president of DSC Communications Corporation, the supplier of the switching computers, said last week that the company had made minor changes in the software provided to several local telephone companies. DSC deemed the changes to be so minor as to not demand the normal thorough testing that switches are subjected to, he said.

That judgment proved faulty. On 26 June, some six million customers in Washington and Baltimore lost their local telephone service for more than six hours, and on the same afternoon three million residents of the Los Angeles area were without a local telephone service for three hours. On 1-2 July, Pittsburgh experienced two failures lasting a total of nine hours, and San Francisco was saved from a massive telephone blackout by telephone company engineers who shut down a misfiring switch before it could trigger other failures.

The pattern of the crashes was nearly identical. A minor failure in one switching computer led to a growing series of error messages being exchanged between switches, until the error messages — which have priority over telephone calls — crowded out normal telephone traffic.

At first, officials from DSC and the various local telephone companies doubted that the software could be to blame, because DSC provided different versions of the software to the different telephone companies, and because, while the software was shipped out from the beginning of March and installed at various times, the breakdowns struck within a matter of days in widely separated areas of the country.

It now seems that each version of the software had the same bug, but the near simultaneity of the crashes must be chalked up to coincidence.

DSC provided software patches that it says should prevent any recurrence of the telephone failures, but concern remains about the vulnerability of increasingly complex phone systems to breakdowns as well as to deliberate sabotage — something that telephone company officials do not believe was a factor in this case. Last week, for instance, the Federal Communications Commission said that it has decided to increase its own research into the reliability of telecommunications networks.

Robert Pool

ITER work is shared out

Washington

AT the third attempt, the four international partners planning to build the world's most advanced tokamak fusion reactor have agreed on a site from which to coordinate the machine's design. Assuming that each of the United States, the European Communities (EC), Japan and the Soviet Union decide to continue funding the project, work on the \$1,000 million six-year engineering design phase for the International Thermo-nuclear Experimental Reactor (ITER) should begin before the end of the year, led by a director based in San Diego, California.

But to forge agreement at a meeting last week in Reston, Virginia, negotiators had to abandon the idea of concentrating the international design team at a single site. Attempts to agree on a single site at meetings in Japan and Germany earlier this year ended in deadlock, with the United States, EC and Japan each arguing that they should be the country to host the design work.

The design team will now be split among three sites: Garching in Germany, Naka in Japan, and the lead site in San Diego. The Soviet Union and Japan will co-chair a council to oversee the project, which will meet in Moscow. The key job of directing the three design teams has been earmarked for a fusion scientist from the EC, making Paul Henri Rebut, who now heads the EC's Joint European Torus fusion project, the current leading contender.

Stephen Dean, president of the industry group Fusion Power Associates, believes that the division of work among sites will not be a major problem. A large proportion of the design work was always intended to be delegated to scientists working in their home countries, he points out.

Dean says that an equitable agreement, allowing each of the partners to claim a share of the prestige associated with hosting the project, was important "to maintain political enthusiasm" for ITER in each of the participating countries. Given that commercial fusion power is thought to lie at least 50 years away, and that the ITER project alone is expected to cost \$4,900 million by the time it is completed early next century, careful nurturing of political support for the project is crucial to quiet nay-sayers during decades of basic research.

The goal of the engineering design phase is to produce a detailed blueprint for the reactor's construction. But, according to Dean, the first year or more may be spent redefining much of the basic 'conceptual design', completed in December 1990 after three years' work by an international team of researchers based in

Garching.

Earlier this year, an independent review of ITER's conceptual design, carried out for the US Department of Energy, recommended that the torus used to contain the reactor's plasma should be built with a higher 'aspect ratio' than was suggested by the Garching team. Essentially, this means building "a skinnier doughnut" to contain the plasma, says Dave Baldwin, from the Lawrence Livermore National Laboratory, who led the Department of Energy review.

Recent experimental findings at the Princeton Plasma Fusion Laboratory and on the recently upgraded Japanese JT-60 reactor, which came too late to influence the ITER design work in Garching, have shown that increasing a tokamak's aspect ratio can improve the retention of heat in the plasma, Baldwin says.

Because fusion researchers are still struggling to achieve 'break even' in tokamak reactors — the point at which the amount of energy produced by fusion reactions equals the energy input needed to heat the plasma — this is considered an important result.

Peter Aldhous

Science societies target station

Washington

LOBBYING to kill funding for the space station Freedom reached a peak last week, with each member of the Senate receiving a letter signed by the presidents of 14 leading scientific societies, urging them not to fund the station at the expense of other scientific projects. The letter warns that, in addition to squeezing the National Aeronautics and Space Administration's space science budget, the escalating costs of Freedom could seriously affect the National Science Foundation's spending in future years.

The signatories to the letter include the American Chemical Society, the American Geophysical Union, the American Physical Society, the American Society of Zoologists and Sigma Xi. But even this concerted effort had little effect. The Senate Appropriations Committee voted to provide \$2,020 million for the station in 1992, the full amount requested by President George Bush, and \$120 million more than agreed on the floor of the House of Representatives last month (see *Nature* 351, 507; 13 June 1991). The full Senate is this week expected also to endorse spending on Freedom.

Peter Aldhous

Sun, sea and shadow

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

A satellite view of the Moon's shadow, travelling across the Pacific Ocean towards Baja California, Mexico, during the total solar eclipse of 11 July. Measurement of this shadow provides a rare opportunity to calculate the precise diameter of the Sun — data used to examine the influence of variation in solar diameter on cli-

mate. Unfortunately, direct observations of the solar corona during the eclipse, made from telescopes at Mauna Kea, in Hawaii, were hampered somewhat by the scattering of sunlight caused by volcanic aerosols released into the atmosphere by the recent eruptions of Mounts Unzen and Pinatubo.

P.A.

Zagury challenges NIH report

Washington

THE controversy surrounding the involvement of National Institutes of Health (NIH) researchers in AIDS vaccine trials led by Daniel Zagury, of the Pierre and Marie Curie University in Paris, is set to deepen this week with the release of a report on the affair from the institutes' Office for Protection from Research Risks (OPRR). The report, which had not been released as *Nature* went to press, but is understood to be sharply critical of the NIH, has drawn an angry 20-page rebuttal from Zagury and his colleague Odile Picard. Their statement rejects the OPRR's complaints as "a jumble of situations and generalizations that are false and contrary to the actual facts". Zagury believes he is being used as a pawn in the internal politics of the NIH.

The OPRR inquiry was prompted by an investigation last year by journalist John Crewdson, of the *Chicago Tribune*, who alleged that trials of putative AIDS vaccines in France and Zaire, involving NIH researchers, failed to comply with US Department of Health and Human Services regulations governing the use of human subjects in clinical trials (see *Nature* 350, 263; 1991). In a letter to the NIH in July last year, Crewdson pointed out that the trials in Zaire involved children, in clear violation of US regulations, and alleged that subjects made available to Zagury by the Zairian authorities included political prisoners. Zagury's co-workers, before the NIH suspended the collaborations in February this year, included Robert Gallo, of the National Cancer Institute, and Bernard Moss, of the National Institute of Allergy and Infectious Diseases.

In his rebuttal of the OPRR report,

Zagury says that the office made no effort to substantiate Crewdson's allegations. In any case, he argues, the French and Zairian trials were not done in collaboration with NIH researchers, so were not bound by US regulations. Although Gallo was named as a co-author on papers describing the trials, Zagury says that he was not involved in their design or implementation. Gallo's role was limited to "an intellectual exchange" over the results and providing comments on the manuscripts, Zagury says.

The OPRR has also examined the use by Zagury of a recombinant vaccinia virus, containing genetic material from HIV, supplied by Moss for use in animal experiments. Zagury admits that he did use this strain once on eleven subjects in 1986, before Moss wrote asking Zagury not to use the strain in human clinical trials. But again, Zagury denies that this constitutes a true collaboration with Moss.

But Zagury's protests have cut little ice at OPRR. "We have heard endless and fruitless arguments about what is or is not collaboration," says office director Charles McCarthy. But that is beside the point, he says. OPRR's concern, says McCarthy, is whether the NIH obeyed its own procedures in deciding whether the research projects in question were subject to US regulations. Standard NIH procedure demands that researchers involved in clinical trials in foreign countries should submit an abstract describing the work to a review board at their own institute. It is up to this board to decide if the work must comply with US regulations. But for the collaborations with Zagury, this was rarely done, says McCarthy.

Peter Aldhous

MEDICAL RESEARCH

Boost for women's health

Washington

BARELY noticed amid the row over spending on the space station, research into women's health is emerging as the big winner in US science spending. As the 1992 budget appropriations bill rumbles through Congress, tens of millions of dollars are being added for women's health research, on top of a presidential budget request for the National Institutes of Health (NIH) that was already seen as generous.

Last year, women's health research became a hot political issue, after a General Accounting Office report found that women were badly under-represented in clinical trials sponsored by NIH (see *Nature* 345, 754; 1990). But a

high-profile campaign by members of the Congressional Caucus for Women's Issues to raise the priority of women's health research at NIH now seems to be bearing fruit.

The additions to the presidential budget request, which are expected to yield an extra \$70 million or so for women's health projects by the time the 1992 budget is finalized, will allow a start to be made on the much-publicized Women's Health Initiative of Bernadine Healy, the new director of NIH.

Projects include studies into breast and ovarian cancer, and a ten-year study into the health of a sample of several hundred thousand women.

Peter Aldhous

Genome researchers go hog wild

London

TO the long list of animals that have their own genome project, add one more: the pig. In April, researchers at 16 European laboratories began PiGMAP — a three-year pan-European project to create a physical and genetic map of the pig genome. Supported by about 6 million ECU of European Communities and national funding, the project joins genome initiatives on worms, yeast, mice, rice and the

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Model species?

common weed in taking advantage of the new mapping technology being developed for the human genome initiative. Pigs are a perfect species to map because they breed rapidly and their 18 chromosomes are of widely varying lengths, which are easy to sort by mechanical means, says University of Edinburgh biologist Alan Archibald, one of the project co-ordinators. PiGMAP's initial aim is to produce a porcine genetic map with about 150 markers, spaced 20 centimorgans apart, with which researchers can find genes that control such traits as growth speed and litter size.

C.A.

EC targets zoos for crackdowns

London

HALF of the 1,000 zoos in countries of the European Communities (EC) could be closed down under a proposed EC rule for zoo animals passed last week. The rule closes a loophole in the 1982 Wellington Convention prohibiting the importation of endangered species to inadequate facilities, by specifying for the first time what, broadly, makes an adequate facility. Unlike the language of the convention, the new rules require that the "behavioural, social, and biological" needs of the animals be fulfilled, but they leave the specifics up to the member states. The rules require accepted standards of husbandry and veterinary care, and allow only "scientifically based breeding" — a criterion that one particular zoo owner in Belgium, who last year tried to cross a lion with a tiger, would have failed to meet. The activist group ZooWatch has estimated that, of the 1,050 zoos in Europe, as many as 500 would fail the test.

C.A.

Bridging an old rivalry

Tokyo

JAPAN's two space agencies, the National Space Development Agency (NASDA) and the Institute of Space and Astronautical Science, may join forces for the first time ever to develop a large solid-fuel rocket, if a proposal put forward by NASDA this week to the Space Activities Commission is accepted. The proposal indicates a warming in relations between the two former rival space agencies that may strengthen Japan's space industry and space science programme.

Japan is the only country in the world that has two independent government-run space agencies each with its own launch sites and rockets. The smaller but older Institute of Space and Astronautical Science began its ventures into space in the mid-1950s with the launch of a small solid-fuel rocket called Pencil. By last year, the institute, working on a shoestring budget from the Ministry of Education, Science and Culture, managed to launch Japan's first probe to the Moon with one of its domestically developed solid-fuel rockets (see *Nature* 343, 403; 1990). And the little institute has had a string of successes in its pursuit of the scientific study of space over the past few decades.

NASDA, which was established by the Science and Technology Agency in the late 1960s and now has nearly ten times the annual budget of its academic counterpart pursued an entirely different path. It imported expensive US rocket technology and used it to launch mainly applications satellites, such as weather and communications satellites. The agency also launches some Earth observation satellites for scientific purposes.

Over the past decade, NASDA has been reducing its dependence on US rocket technology by developing Japanese-made liquid-fuel engines, such as that in the second stage of its current H-I rocket. By 1993 the agency hopes to launch its next-generation H-II rocket, which will be made entirely made in Japan.

It is NASDA's gradual switch to domestically developed rocket technology that has created the opportunity for the two agencies to develop a rocket jointly. In the proposal submitted this week, NASDA calls for the solid-fuel booster rockets of the H-II to be combined with the second stage of the academic space institute's M-3SII, the rocket that sent a probe to the Moon last year.

The hybrid rocket could be used to launch medium-sized, one-ton satellites into low Earth-orbit at very low cost. NASDA is also keen to use the new rocket for supersonic, upper-atmosphere

test flights of the agency's proposed unmanned space shuttle HOPE, which will service the Japanese module of the planned US Space Station (see *Nature* 351, 680; 1991).

Jun Nishimura, director general of the smaller agency, says the NASDA proposal is a "very interesting possibility". The solid-fuel boosters for the H-II were developed by Nissan motor company using the institute's technology (Nissan also makes the institute's rockets). And so combining the two would be quite logical, says Nishimura.

But the institute jealously guards its independence and is eyeing the NASDA proposal warily. Relations between the two space agencies were strained for many years because, in an unusual political deal in the late 1960s, the Ministry of Education, Science and Culture and the Science and Technology Agency agreed to limit the tail diameter of the institute's rockets to a maximum of 1.41 metres so

that the institute did not compete with the newly formed NASDA.

The size limit was recently lifted, opening the way for the institute to begin developing a much larger rocket, the M-V, which will be able to lift 2-ton satellites into low Earth orbit.

Many Western observers comment on the seeming inefficiency of having two space agencies. But the institute's scientists argue that the division of roles has enabled them to run a very cost-efficient and low-budget space science programme unmatched by any other space agency in the world. They have no intention of merging with the larger agency, they say.

The sharing of rocket technology, however, would expand the choice of launch vehicles and allow greater flexibility in the size of payloads, and so the idea is being seriously considered by the institute, although a decision will take many months. No matter what the outcome, NASDA's proposal does seem to herald a new era of cooperation between the two agencies.

David Swinbanks

NUCLEAR ENERGY

An urgent search for power

Tokyo

TOKYO Electric Power Company, the world's largest private electric utility company, last week turned on all 13 of its nuclear power generators to try to cope with a surge in demand for electricity during Japan's steaming hot summer. The company will also rekindle two fossil-fuel thermoelectric power plants to meet a demand that is expected to reach the brink of the power company's capacity.

The summer energy shortage is drawing attention here to critical problems that Japan faces in expanding its energy sources, nuclear power in particular.

In recent years, Japan's energy consumption has defied government predictions and grown at about five per cent a year, twice the projected rate. Much of the increased demand is coming from private households that have recently bought energy-guzzling air conditioners.

Last summer, Tokyo Electric came within a few per cent of its maximum electric power generating capacity during peak demand periods. The company was able to buy extra capacity from neighbouring power companies to maintain an adequate safety margin, but the option to buy surplus electricity is much more limited this year at a time when demand is expected to be even greater.

In February, the Tokyo power company's largest neighbour, the Kansai Electric Power Company, suffered a major setback: one of its oldest nuclear power reactors was shut down during an emergency (see *Nature* 349, 557; 1991). The

500,000-kW Mihama reactor is not expected to come back on line for at least another three years. And shortly after the Mihama accident, the Kansai company's Takahama No. 2 reactor had to be also closed down. As a result, the company is also struggling to meet energy demands and has no surplus power to sell to its energy-hungry neighbour.

Both companies are looking to fossil-fuel plants to cope with the energy crisis. Tokyo Electric will reactivate two thermoelectric power generators in Chiba prefecture next to Tokyo to help cope with anticipated demand this summer. Kansai Electric is planning to build two large thermal power plants fuelled with liquefied natural gas and another powered by coal by the end of this century.

The move back to fossil fuel has focused attention on the problems of finding new sites for nuclear power plants. Japan's Ministry of International Trade and Industry has called for 40 new nuclear plants to be built by 2010. Eleven plants are under construction, but there is growing local opposition to nuclear power, and the government and industry have been unable to allocate any new sites in recent years, despite huge financial incentives for local governments to accept nuclear power plants.

Switching back to fossil fuel is not a viable alternative in the long run because Japan has pledged to hold its output of carbon dioxide emissions to the 1990 level after the year 2000.

David Swinbanks

EC putting bite in its green bark

London

By day the Eurocrats of the European Communities are the green champions of Europe, passing more environmental legislation in the past three years than in the previous two decades. But by night, when they return to their Brussels homes, they are among the EC's worst polluters. One hundred per cent of the sewage in the EC capital flows raw and untreated into the North Sea. This, they admit, does not look good. Worse, it is a glaring reminder of the principal problem with the European Commission's environmental legislation. Its laws may be environmentally stringent, comprehensive and far-ranging, but they are also widely flouted.

Despite an aggressive record of green legislation — 450 regulations are already in effect, and some 90 others are set to join them this year — the Commission has a reputation for toothlessness. Beyond Belgium's sewage, there are Britain's seashores, rivers and drinking water, Italy's air and Greece's birds. More than 400 cases of non-compliance are now being argued in the European Court. Several member states have failed to ratify — and make national law — even half of the EC directives.

The problem is that, while the European Commission has become very good at passing rules, it has given itself precious little with which to enforce them. It has no inspectors to find out if its directives are being followed, and no comprehensive monitoring programme to gauge the effect of the rules that are being kept. Typically, the only way the Commission learns that a country is violating a directive is when some citizen writes a letter of complaint. Even then, all the Commission can do is

file a lawsuit with the European Court, a process that often takes three or more years; it has no power to levy fines.

"In general, the implementation [of EC environmental regulations] has been slow, and it has been patchy," says Ted Bennett, head of the EC's Industry and Environment directorate.

Earlier this year, the Communities' Environment Commissioner, Carlo Ripa di Meana, announced plans for the EC's first force of environmental inspectors — 'green police' — who would inspect member states to see if they are complying with EC laws. The inspectors should be able to levy fines, Meana said.

EC officials are also looking to economics to help stem the leak in their enforcement dike. Last year, after the European Council of Ministers declared that standards alone were no longer enough and called on the Commission to use its economic leverage to bring member states into line, an EC working group laid out five economic 'tools' to encourage environmental compliance: subsidies, deposit-refund systems, emissions trading, taxes and fines.

Community pressure for a tax on carbon dioxide emissions quickly brought the debate on economic measures to the fore, and they are expected to figure prominently in a major revision of the EC Treaty later this year. EC officials say that subsidies will probably be used only where compliance would cause serious economic disruption, such as the possible consequence of reducing fertilizer use in farming.

Taxes, apart from those on carbon dioxide, are planned mostly to generate revenue to subsidize other enforcement

systems, such as inspectors. Meanwhile, emissions-trading schemes are expected to be introduced to help control release of sulphur dioxide, nitrogen oxide, volatile organic compounds and particulates.

But the hurdles that still lie between the EC's rules and practised reality are considerable. After 20 years of EC environmental legislation, the compliance barriers that remain include the following.

■ **Cost of compliance:** If the EC rules were just an endorsement of the status quo, compliance would be no problem. But in most cases they go beyond existing national legislation in many of the member states, and bringing those countries into line is not cheap. In Britain, for example, the cost of implementing just one — reducing sulphur levels in gasoline — will cost refineries some £270 million. Upgrading sewage treatment to conform with EC regulations will cost the UK £1,500 million over the next decade.

■ **National politics:** "It's actually quite hard to write a national law that will follow an EC directive," says one UK environmental official. In Belgium, for example, the constitution has a federal-state separation clause that makes it very difficult to write federal legislation without violating state rights.

■ **Lack of good data:** Environmental monitoring among the EC member states is still patchy, suffering from a mishmash of measuring techniques and differing national reporting policies. Without a clear picture of what is being released where, the EC can hardly find trouble spots. Last year, it created a European Environment Agency to start a comprehensive monitoring and data gathering programme, but the agency (see below) has been tangled in politics since its inception and has yet to get a home.

Christopher Anderson

New environmental agency hijacked by politics

THE future of the new European Environmental Agency has been held hostage to EC politics since its announcement last year. France insists that there will be no decision on a home for the agency nor further decisions on its purpose until the EC states resolve the question of where to locate the headquarters of the European Parliament, a debate that is already a half-decade old and with no end in sight.

That is unfortunate, because the environmental agency's mission — to compile and distribute data about Europe's environment — is critical. Environmental data plotted on a map sometimes shows political boundaries — not because the air is different across the borders, but because the measuring systems are. It is hard to make en-

vironmental policy without accurate information.

A working environment agency could provide the EC with just that. Approved in March last year with funding of about 5 million ECU, the agency does actually exist, in a way. It has a director and a small staff in Brussels. But until it gets a real headquarters and its marching orders, it can do little but farm out small data-gathering contracts to some of the institutions vying to be its home.

The agency is expected eventually to focus on data collection and dissemination, acting as an 'interface' between basic environmental research and environmental policy.

Eleven of the 12 member states have put in bids for the agency, including the

Netherlands, whose National Institute of Public Health and Environmental Protection (RIVM) is already well on the way towards compiling environmental data for the entire continent.

EC environment minister Carlo Ripa di Meana is fighting hard to have the agency 'unlinked' from the question of the Parliament's location, but he has so far met with little success. In the meantime, Italy, France and the United Kingdom have all announced plans for agencies of their own, to monitor and research their domestic environments. Ideally, these agencies should be working with the European Environment Agency to set common data policies. But with its future still in the air, there is not much the agency can do to help. **C.A.**

Responsibility and Weaver *et al.*

Paul Doty, Mallinckrodt Professor of Biochemistry Emeritus at Harvard University, points to the scientific community's responsibility in the case of Weaver *et al.*

Each profession establishes by its practices its own ethic. If the leadership of the scientific community, especially the biological sector, embraces a common standard of how research should be carried out and reported, the time has come for it, as well as the wider community, to examine the case of the *Cell* paper by Weaver, Reis, Albanese, Constantini, Baltimore and Imanishi-Kari (*Cell* 45, 247; 1986) and to assess the extent to which this standard has been met or compromised. The community has before it more than a thousand pages of Congressional testimony, hundreds of pages of comment, the leaked draft Report of the Office of Scientific Integrity (OSI) and the extensive exchanges in *Nature* and elsewhere that this draft report has stimulated.

Such an assessment will require more time than many are willing to invest; yet unless some do, the community's reaction to this important case will be indecisive, dictated by personal affiliations and superficial understanding. I record here my own reactions after considerable study in the hope that it might stimulate others to examine the matter. So far, attention has been focused primarily on the validity of the data reported and the various means taken to investigate possible misconduct. This has overshadowed the question of whether the account given by the authors themselves represented a departure from the normal standards of research, which puts the uncompromising search for truth first and, if so, whether this reflects a more general lowering of the standards by which research should be carried out and reported.

To be sure, Dr David Baltimore, the most senior of the authors, has apologized for some aspects of his behaviour — five years after he was informed of serious shortcomings in the work and two months after severe condemnation in the draft report of OSI. However, the apology, although welcome, does not erase from the record the behaviour that occurred and was defended over five years and omits mention of many other actions (M. O'Toole *Nature* 351, 180; and D. Baltimore *Nature* 351, 341; 1991). Moreover, until the final OSI report is released, we will not know the extent to which the opposing views of the authors of the *Cell* paper will have affected final judgements. And the acceptance by the OSI draft report of compelling evidence for falsification of data may not be settled until there has been a court review. But, in my view,

the case for egregious departure from the usual standards of carrying out and reporting research stand independent of these remaining conflicts. The same applies to the succession of failures of reviews of the paper and the procedures used to address complaints against it once serious questions had been raised.

Consider first a few of the lapses in scientific standards seen in the actions of various authors. The recording of data, especially by Dr Thereza Imanishi-Kari, was so sloppy as to insult the scientific method. Reviewing the case strictly from Baltimore's published account reveals at least four lapses from what have been the traditional standards of science. He (1) failed to examine critically the quality and sufficiency of the data before publication; (2) failed to examine the data and report the possibility of error after serious criticisms were made; (3) instead organized an attack on his critics and discouraged publication of their views; and (4) did not subject the conclusions to further tests or check the reproducibility of what had been reported in a timely manner.

One example of departure from standard practice was the elimination of an embarrassing, albeit weak, RNA band in the reproduction of a northern blot by under exposure. The draft report calls this "misleading" and states that the explanation of the author (Weaver) "does not excuse his failure to indicate in the paper the existence of possible" expression of the transgene.

Baltimore's attitude towards the responsibility of authors checking the reliability of their own data is a critical departure from common standards. His claim, in the second congressional hearing, was that this remained for others to do. He even proposed that Congress provide for the repetition of the experiment. To forgo this obligation — to leave to others the responsibility of establishing the validity of what you have published — is not only a fundamental retreat from responsibility but, if it became accepted practice, would erode the way science works. For the cutting edge of science moves forward by building rapidly on what is published on the tentative assumption that it is correct, not by waiting for others to test each paper's validity.

In the several exchanges that have followed the availability of the OSI draft report, there are frequent shadings of response that reveal a continued interest in concealing the whole truth. For

example, in Baltimore's rejoinder (*Nature* 351, 341; 1991) to O'Toole, he states that "she (O'Toole) . . . neglect[ed] to mention that the seventeen pages have been proven to be irrelevant". However, the OSI draft report describes the way in which the data reported in the 17 pages was presented in the *Cell* paper as "not defensible". Therefore, the 17 pages are hardly irrelevant and it is misleading to suggest that O'Toole should have said they were.

This pattern of behaviour stands in deep contrast to the traditional view that authors of scientific papers have a special obligation to be responsive to criticism and to test their work from every possible angle — to pursue the truth relentlessly.

The late Richard Feynman captured this spirit when he wrote, "It's a kind of scientific integrity (that is at stake) a principle of scientific thought that corresponds to a kind of utter honesty — a kind of leaning over backwards . . . (to) report everything that you think might make (an experiment) invalid". It is essential that the other issues involved in the *Cell* paper controversy are not allowed to overshadow this key tenet.

Having known Baltimore over the years, I find it painful to dwell on this matter because it affects only a very small part of his work. But to let this lapse pass in silence would be to condone it and to fail to recognize the very different standards of his other work as well as the harm that has come from the posture he has taken. It can only be hoped that in the future he will see assessments such as this as an effort to act in a manner that he would support in other circumstances.

The shortcomings in this case go well beyond the behaviour of the authors to the procedures within the scientific community for insuring against publication of inaccurate or misleading data or its prompt correction. A major failure must be laid to the investigating committees appointed by the institutions involved. By an exhaustive review of the data, reporting promptly a clear record of the issues and recommendations in a public report, they would have provided decisive guidance that would have terminated this case. Such committees, properly selected and properly charged, have worked successfully elsewhere to recall or withdraw fraudulent or erroneous papers. In this case, the committees at both Tufts and Massachusetts Institute of Technology (MIT) consisted solely of close colleagues of various of the authors, and in the case of MIT consisted

of only one person, Dr Eisen. Clearly, others well outside the acquaintances of the authors should have been included. Moreover, the charges to the committees were inadequate, the reports were made many months later and years passed before they became public. Nevertheless, to his credit, Eisen has recently corrected an important misunderstanding in his original report (*Nature* 352, 101; 1991). Finally, the universities did little to protect the rights of the whistleblower.

Once the National Institutes of Health (NIH) took up the investigation, they too faltered. The first NIH investigating committee had to be reconstituted to remove two long-time collaborators of Baltimore, thereby losing valuable time and raising the suspicion of incomplete objectivity. This committee then took the questionable step of accepting supplementary data provided by Imanishi-Kari in lieu of that which was disputed in the original paper. But this action had to be reversed when the Secret Service, brought in at the initiative of Congressman Dingell, presented evidence that some of the supplementary data were compiled after the publication of the *Cell* paper. Meanwhile, Dingell initiated congressional hearings. The first of these probably erred in not having any of the authors testify, but the two subsequent hearings did afford a broad disclosure of all sides of the controversy, exposing a number of points missed by the NIH committee. The newly constituted Office of Scientific Integrity created a panel in the autumn of 1989; and after two years' work it has produced in its draft report the most comprehensive examination that the situation requires.

The scientific community itself has not been very active over these five years. In the first two congressional hearings, a number of scientists were aroused by fears, initiated in part by Baltimore, that inappropriate congressional interference with science was underway, but this has not continued as the seriousness of the charges of questionable behaviour rose. And the National Academy of Sciences did appoint a panel on scientific conduct early in 1991 to recommend procedures for improved self-policing in science but it has not yet reported.

Whatever the faults and virtues of the procedures applied in this case, it is clear that they provide little guidance for the future. Cases of alleged misconduct can not be handled by congressional hearing, which would limit examination to about one case per year. Nor can recourse be made to the courts where the expense and time consumed could not be afforded. It remains to review the several cases where local academic committees have resolved such cases promptly and effectively and see how this method can be strengthened and augmented by special oversight committees such as the OSI might appoint.

But the essence of change must come

within the scientific community by its reassertion of its ability to police itself. In contrast to the pattern of behaviour seen in this case, the scientists making up the OSI panel praised the actions of the whistleblower, Dr O'Toole, as "heroic in many respects" and went on to state that "she deserves the approbation and gratitude of the scientific community for her courage and dedication to the belief that truth in science matters". Yet one sees little other evidence of the scientific community expressing itself accordingly. Unless it does so eventually, its silence can be interpreted as condoning the standards of research and reporting embodied in the Weaver *et al.* paper.

This challenge to readdress the fundamental tenets of acceptable behaviour in science comes at a time when the traditions of the scientific enterprise are under new threats arising from new stresses and temptations. The growth of the enterprise itself with its accompanying bureaucracy, the near cut-throat competition for grants, the possible corruption, on

occasion, of peer review, the growing number of cases of deception in scientific papers, scientists' acquiescence in the increasing avoidance of meaningful review in direct congressional grants for research buildings and projects — all these contribute to the pressure to compromise and erode the high principles of the past. As a result, the scientific community may already be experiencing a gradual departure from the traditional scientific standards; this could be abetted by condoning the behaviour seen in this present case. In this way we risk sliding down toward the standards of some other professions where the validity of action is decided by whether one can get away with it. For science to drift toward such a course would be fatal — not only to itself and the inspiration which carries it forward, but to the public trust which is its provider.

Paul Doty is Mallinckrodt Professor of Biochemistry Emeritus, Harvard University, Boston, Massachusetts, USA

Dissent on forensic evidence

Below we republish extracts from the "minority opinion" by Drs Hugh O. McDevitt and Ursula Storb that accompanied the draft report on Weaver *et al.* by the US NIH's Office of Scientific Integrity.

AFTER noting that they find the sections of the report dealing with the substance of the disputed paper and its internal inconsistencies "accurate and complete to the best of our recollection", the authors discuss the use of forensic evidence in later sections of the draft report "with which we are in serious disagreement".

"In section IV.A statistical analysis of transcribed gamma counter counts reveals that in many instances, handwritten counts do not show a normal Poisson distribution, and display considerable 'spikiness'. We do not agree with either the prominence given the results of these statistical analyses, nor with the conclusions based on these analyses.

"Similarly, in section IV.B, marked periodicity of the counts is found not to be in agreement with a sampling of hybridoma wells which it is purported to represent. However, while this suggests that data from an unrelated experiment were used for pages 102–107 [of the notebook submitted to the Dingell Committee] we cannot accept the statistical evidence as proof. "The second line of evidence presented in sections IV.A & B is much more convincing. The forensic analysis concerning the chemical composition of ink on counter tapes found on [some notebook] pages . . . indicates that a full match for these tapes is not found anywhere after April 1982 in any of the laboratory notebooks used by individuals using the same counter.

"Initially, this might have been explained by an inadequate sampling of

laboratory notebooks of other individuals using the counter in question. However, at the panel's request, numerous additional notebooks were obtained, and it now seems likely that a substantial majority has been examined. Despite this extensive sampling, no other laboratory notebooks had tapes with a full match dated later than April 1982.

"Unfortunately, the panel was not given access to the actual chromatograms which led the Secret Service to make the conclusions referred to above. Examination of chromatographs of similar inks from similar analyses, as well as other available techniques (if chromatographic analysis fails to distinguish two inks) indicates that the methods used are certainly capable of determining identity or non-identity of two ink mixtures. Ultimately, the actual chromatograms need to be examined.

"However, with this reservation, the findings as stated, combined with the other inconsistencies found on the pages referred to above, make it seem likely that the data on these pages are not the result of experiments performed at or near the time stated, but in fact, are data from other experiments performed as much as three years earlier.

"The significance of these data, their relevance to the initial and subsequent investigations, and the corresponding reasons why these data are legitimate targets for the present investigation, are all well described in the report, and we are in agreement with that description.

Detection of human carcinogens

J. Ashby and R. S. Morrod

Rodent cancer bioassays are no longer the most efficient way to detect possible human carcinogens. A more imaginative approach is needed.

How are potential human carcinogens best identified? Early hopes for short-term mutagenicity tests have not been fulfilled; the results of lifetime cancer tests in rodents seem to remain the only reliable indicator. But these tests are both slow and expensive, and there remain many untested chemicals. Further, the validity of carcinogenic responses observed at the maximum tolerated dose of test chemicals has recently been questioned^{1,2}. The time seems to be right for a more imaginative approach to the prediction of potential human carcinogens. Such an approach should include the focused use of standard genetic toxicity tests, urgent evaluation of the new transgenic rodent mutation assays, careful use of empirical markers of non-genotoxic rodent carcinogenesis, and an overall emphasis on mechanistic studies.

Hay recently described in *News and Views*³ current concerns about the interpretation of rodent cancer bioassay data. These are that at the maximum tolerated dose (MTD), tissue toxicity may induce cell divisions (mitogenesis) that themselves may provoke the DNA mutations from which tumours eventually develop^{1,2}. Thus, chemicals labelled carcinogenic by these criteria may not present commensurate hazard to people exposed to lower, non-toxic concentrations. Another concern is that rodent bioassays are devoted almost exclusively to synthetic chemicals. Although Peto has suggested that synthetic chemicals probably make a negligible contribution to the overall incidence of human cancer⁴, there is an absolute need to evaluate new chemicals for potential carcinogenesis^{5,6}. A particularly serious concern is that if rodent tests are providing misleading data, this undercuts regulatory initiatives in this area of human health protection.

During the past 15 years a picture has emerged⁷⁻¹⁰, indicating that some carcinogens are both genotoxic (reactive to DNA) and toxic. A growing number of non-genotoxic chemicals are also being identified that induce selective carcinogenic activity in rodents at or close to the MTD and whose activity in humans cannot always be assumed.

The different types of rodent carcinogen can be illustrated by three chemicals tested by the US National Toxicology Program (NTP) (Fig. 1). The first, glycidol, is genotoxic and carcinogenic to

many tissues in rats and mice, and as such is representative of most of the established human carcinogens^{11,12}. The other two carcinogens in Fig. 1 are non-genotoxic and have highly selective carcinogenic effects. It is thought that these last two carcinogens may be active by virtue of toxic effects they elicit specifically in rodents^{1,2}, that is, there would be no corresponding human hazard upon exposure. This cannot be assumed, however, without mechanistic data¹³⁻¹⁵. The disparity between the carcinogenic activity of the first and the second two chemicals suggests that the classification 'rodent carcinogen' is too imprecise, and that there may be more efficient ways of anticipating such selective non-genotoxic carcinogenic effects than the conduct of MTD tests.

As a consequence of these considerations, we believe it is no longer efficient simply to conduct MTD carcinogenicity tests on the many thousands of chemicals to which humans are exposed, if for no other reason than it would take several centuries to complete the task. We suggest an alternative approach: to conduct short-term tests for effects associated with rodent carcinogenicity, and to conduct limited cancer tests when more precise knowledge about carcinogenicity of a chemical is required (Fig. 2).

The first step is to inspect the chemical structure of each agent for sites of DNA-reactivity¹⁶, followed by assessment of genotoxicity using *in vitro* tests and, if necessary, short-term rodent tests^{17,18}.

This will identify potential genotoxic carcinogens. The few uncertainties remaining concerning the use and interpretation of genetic toxicity tests can be resolved once the concept of non-genotoxic carcinogenesis is accepted. The main topics of discussion in genetic toxicology are the use of *in vivo* tests to separate *in vitro* genotoxins that are rodent carcinogens from those that are non-carcinogenic to rodents, and the use of complementary genotoxicity assays to detect the few *Salmonella*-negative genotoxins, such as benzene¹⁶. But the vast majority of classical human and rodent chemical carcinogens (benzo[*a*]pyrene, benzidine, dimethylnitrosamine, and so on), together with most of the NTP two-species carcinogens, are overtly genotoxic *in vitro* and *in vivo*. Genotoxicity observed for a chemical in short-term (1–3-day) rodent tests does not, however, provide data on likely carcinogenic potency or organotropy — criteria that are important for accurate human risk assessment. Determinants of carcinogenic potency and organotropy become effective only during the course of a bioassay and are probably associated with subtle toxic responses to protracted dosing. In that context, the newly developed transgenic rodent mutation assays should be important because they could measure species/strain-specific amplification of spontaneous or chemically induced mutation incidences at varying times during a bioassay.

The next step in our scheme (Fig. 2) is

CAS No NTP Tech Report No (Year)	Chemical Name	Structure or trivial name (alerting substructure in bold)	Structural alert	Salmonella assay response (Zeiger)	Tumour site	Tumour data identified in Summary of NTP Technical Report											
						% Tumour-bearing animals											
						Rats						Mice					
						Male		Female		Male		Female		Male		Female	
C	L	H	C	L	H	C	L	H	C	L	H	C	L	H			
556-52-5 374(1990)			+	+	Tunica vaginalis Mammary gland Brain Oral cavity Stomach Skin Zymbal's gland Clitoral gland Thyroid Haemopoietic system Harderian gland Uterus Subcutaneous Liver Lung	6 6 0 2 2 0 2 2 0 0 26 0 0 48 26	68 16 10 4 10 6 12 18 2 2 26 2 2 62 22	78 14 12 14 22 8 12 24 6 40 6 50 70 42									
Glycidol																	
78-42-2 274(1984)			—	—	Liver												
Tris(2-ethylhexyl)phosphate																	
85-68-7 213(1982)			—	—	Haemopoietic system			12	14	36							
Butyl Benzyl Phthalate																	

FIG. 1 Rodent carcinogenicity of the genotoxin glycidol compared with the selective rodent carcinogenicity of two non-genotoxins. Data reported by the NTP. C, Control group; L, low-dose group; H, high-dose group. No entries in a box, no carcinogenic response recorded. (Data from ref. 16.)

to evaluate the remaining non-genotoxins for a range of toxicities associated with non-genotoxic rodent carcinogenesis. Agents found to be inactive are probably not carcinogenic. Fourteen short-term (less than 10-day) indicators of non-genotoxic rodent carcinogenesis are shown in Fig. 2 (refs 9, 19–23). None of these has been established as a definitive predictor of the appearance of tumours, but all are associated, to varying degrees, with tissue-specific non-genotoxic rodent carcinogenesis and each has the potential to become a specific indicator. Until mechanistic links with carcinogenesis are established, however, it is safer to associate the absence of each of these effects with probable non-carcinogenicity, rather than vice versa. Butterworth, for example, has emphasized that mitotic activity in the liver cannot be regarded as indicative of non-genotoxic hepatocarcinogenesis because so few non-carcinogens have so far been studied⁹; nonetheless, we suggest that the absence of mitotic activity in the liver provides a strong indication of the probable absence of non-genotoxic rodent hepatocarcinogenesis. Many of these indicator events could be monitored in the same animal.

Many issues have yet to be understood about non-genotoxic carcinogenesis. For example, the control incidence of mouse liver tumour varies between 0 and 58% in 41 NTP bioassays where these tumours are induced by a test chemical. Gaining an understanding of the causes of this variability in spontaneous tumour incidence, and how chemicals might affect the underlying genetic control mechanisms, should contribute significantly to an explanation of the many non-genotoxic mouse liver carcinogens defined over the past decade¹⁶.

Developing and refining the test sequence shown in Fig. 2 would allow limited rodent carcinogenicity bioassays to be used to confirm genotoxic rodent carcinogenesis, non-genotoxic rodent carcinogenesis and non-carcinogenesis. Given the preliminary tests, the standard two-species/two-sex carcinogenicity bioassay would therefore be needed much less often. Some predictions may be sufficiently strong to make the conduct of lifetime carcinogenicity bioassays completely unnecessary. Note that 96 per cent of the NTP carcinogens would be detected as positive using a restricted bioassay of male rats and female mice²⁴.

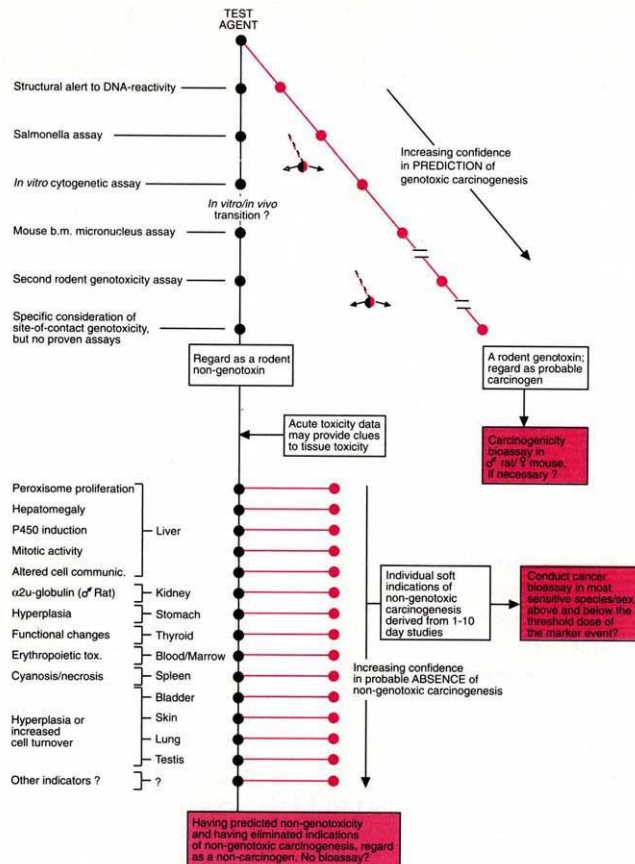


FIG. 2 Proposed testing strategy that integrates current predictive techniques with specific and limited rodent carcinogenicity bioassays. Evaluation for genotoxicity normally ceases after adequate negative studies *in vitro*, or after a positive response *in vivo*. Black circles, negative response; red circles, positive response; red/black circles, heterogeneous response or response of doubtful biological significance.

Data obtained from the non-genotoxic marker assays may also yield valuable dose-response information (including an indication of a threshold dose), which may provide valuable information for human risk estimations.

Nothing in our scheme is new; we are simply integrating and balancing seemingly unrelated techniques in a holistic approach to carcinogen prediction. For example, the fact that genotoxicity assays alone cannot be used accurately to predict rodent carcinogenesis sometimes leads to their total neglect. We believe that these tests can be used to estimate the potential for genotoxic carcinogenesis without having to corre-

late perfectly with it. Similarly, we suggest that the non-genotoxic indicator events shown in Fig. 2 are not directly carcinogenic, but are part of a modified environment in which some tissues of some sexes of some species are provoked into an increased (or decreased) incidence of tumours. Some of the indicators of non-genotoxic rodent carcinogenesis shown in Fig. 2 may be warnings to rodent-specific carcinogens²², or may be of no value. But such knowledge can only come from testing assumptions about their prospective use²⁵.

In cases where a chemical of completely unknown biological activity is being tested for carcinogenic activity, use of the MTD seems to be justified. However, as emphasized by Ames and Gold^{1,2}, toxicity observed at or close to the MTD may produce an exaggerated carcinogenic response for genotoxic and non-genotoxic carcinogens, leading to exaggerated risk estimations for low-level human exposure.

Ames and Gold^{1,2} are concerned by the facts that two-species rodent bioassays are devoted almost exclusively to the evaluation of synthetic chemicals, and that MTD sometimes exaggerates rodent carcinogenicity. We are concerned that such bioassays are used as the first rather than the last resort when evaluating an agent's carcinogenic potential. Reconciliation of these two separate concerns could be achieved by greater use of predictive techniques and the consequent release of part of the resources used for rodent cancer bioassays to studying mechanisms of carcinogenic action and evaluation of the carcinogenicity of selected natural and endogenous chemicals. □

J. Ashby and R. S. Morrod are at the ICI Central Toxicology Laboratory, Alderley Park, Macclesfield SK10 4TJ, UK.

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Trying an authorship index

SIR — Few issues in scientific life can now match authorship of collaborative work for its potential to distract and destroy. The use of bibliometric indices as performance indicators places great weight upon uncertain foundations. How does one compare senior versus junior, staff member versus visitor, money versus time, or backache versus headache versus heartache?

The unit in which I work uses a set of formal rules based upon a simple points table. The maximum score possible is 100 points. Each potential author is awarded the highest realistic score in each category; whoever achieves a total of 25 points is offered joint authorship in rank order of total score. In the event of ties, recent near-misses are considered; if none exists, alphabetical order is used.

The scheme is used mainly for ex-

perimental papers in plant ecology. A variant for theoretical studies has a 15-point scale for data-capture and a 25-point scale for specialist input. However, we have avoided too much tinkering because simplicity and generality are important goals. Preliminary experience with these rules has been encouraging — perhaps readers may wish to test them for themselves?

RODERICK HUNT

NERC Unit of Comparative Plant Ecology,
Department of Animal and Plant Sciences,
The University,
Sheffield S10 2TN, UK

COAUTHORSHIP SCORING SYSTEM

INTELLECTUAL INPUT (planning/designing/interpreting)	
No contribution	0
One detailed discussion	5
Several detailed discussions	10
Correspondence or longer meetings	15
Substantial liaisons	20
Closest possible involvement	25

PRACTICAL INPUT: DATA-CAPTURE (setting-up/observing/recording/abstracting)	
No contribution	0
Small contribution	5
Moderate indirect contribution	10
Moderate direct contribution	15
Major indirect contribution	20
Major direct contribution	25

PRACTICAL INPUT: BEYOND DATA-CAPTURE (Data processing/organizing)	
No contribution	0
Minor or brief assistance	5
Substantial or prolonged assistance	10

SPECIALIST INPUT FROM RELATED FIELDS	
No contribution	0
Brief or routine advice	5
Specially-tailored assistance	10
Whole basis of approach	15

LITERARY INPUT (contribution to first complete draft of manuscript)	
No contribution	0
Edited others' material	5
Contributed small sections	10
Contributed moderate proportion	15
Contributed majority	20
Contributed virtually all	25

Proof negative

SIR — In his Commentary 'Does climate still matter?' (*Nature* 350, 649–652; 1991), Jesse H. Ausubel argues that "we seem to be 'climate-proofing' society, making ourselves less subject to natural phenomena". He notes that this is heartening and that it "would seem sensible to maintain this course and not to revert to reliance on . . . technologies . . . that are more sensitive to climate".

Curiously, he fails entirely to recognize that climate-proofing has depended

heavily on the increased use of fossil fuels. In order to limit emissions, it is widely argued that we must ultimately reduce global use of fossil fuels and increase our dependence on energy sources such as biomass fuels, wind, hydropower and solar energy. Yet doing so may increase our vulnerability to climate change.

Ausubel himself notes that "a system of energy from wood and hay was more climatically sensitive than one reliant on oil and natural gas". He adds that water and wind power "are, of course, more sensitive to climate".

The threat of climate change confronts us with not one challenge but two. We must not only reduce emissions but also our vulnerability to changing climates. As in Ausubel's analysis, these are often treated as separate problems. Consequently, an important dilemma has been largely overlooked: the shift away from fossil fuels may increase our vulnerability to climate change at precisely that moment in history when we need to "climate-proof" ourselves.

NICK SUNDT

1347 Massachusetts Avenue SE,
Washington, DC 20003, USA

Sampling error?

SIR — Others have drawn attention to serious anomalies in the procedure undertaken for radiocarbon dating the Shroud of Turin (Damon *et al.* *Nature* 337, 611–615; 1989).

Your readers should know that anxiety about the procedures followed has been heightened by a recent declaration of Professor Wolfli, one of the 21 co-authors of Damon's report. In a short interview published in the French monthly journal *Contre-Reforme Catholique*, Wolfli asserts that the size and weight of the shroud samples mentioned in Damon's paper were erroneous. According to the French journal, he declared: "Nobody (among the authors) has seen this error. We were under pressure, but that is not an excuse." So far, this statement has not been challenged in any way by the first author, Damon. Because sampling procedures have always been regarded as critical in the dating of the shroud, this situation is most disturbing.

This unique archaeological artefact deserves more serious attention. Logically, scientists who question this procedure should be allowed to review the original records, including the videotapes recorded during sample collection in Turin in April 1988. How can this be achieved?

PIERRE BUSSON

Lineberger Cancer Research Center,
University of North Carolina,
Campus Box 7295, Chapel Hill,
North Carolina 27516, USA

No friction

SIR — The news item "Friction continues over costs" (*Nature* 351, 594; 1991) greatly overstates the difficulties in the interaction between the European particle physics centre in Geneva (CERN) and the Superconducting Super Collider Laboratory in Texas (SSCL). In fact, the Memorandum of Understanding that we signed in April is working well. Scientists from our laboratories are working together effectively on matters of accelerator design, technology development, and other issues of mutual interest and concern; and we expect the cooperation to continue.

Both of us have always recognized and acknowledged the complementary nature of the programmes of CERN and the SSCL. CERN is one of the world's major centres for high-energy physics; the SSCL is becoming one. Outstanding scientists from around the world work at the two laboratories, and their cooperation can only enhance the quality of scientific work in this exciting and rapidly developing field.

The frontiers of physics are always characterized by some degree of competition among scientists striving to make critical discoveries, but this competition fosters and improves scientific programmes and should not be confused with disruptive "friction".

CARLO RUBBIA
Director-General

CERN, CH-1211, Geneva 23,
Switzerland

ROY SCHWITTERS
Director

SSCL,
2550 Beckleymeade Avenue,
Dallas, Texas 75237, USA

Volcanoes' volatile behaviour

Jonathan Fink

THE recent eruptions of Mount Pinatubo in the Philippines and Mount Unzen in Japan have caused local residents, civil-defence authorities, politicians, reporters and US military officials to ask three of the most fundamental questions about volcanoes: why do they start erupting, what controls the violence of those eruptions, and when do the eruptions stop being dangerous? A new study by geophysicists Claude Jaupart and Claude Allègre¹ uses an analysis of gas solubility in molten rock together with observations of several explosive volcanoes to address these questions quantitatively. The paper challenges a prevalent view that the single most important factor controlling the explosiveness of eruptions is the amount of gas dissolved in the source magma body and argues instead that it is the ease with which this gas can escape from the magma as it rises to the surface that influences whether an eruption will be violent. The analysis explains why the appearance of a lava dome in the crater of Mount Pinatubo may be an early sign that explosive activity is coming to a gradual close. However, it also warns that the growth of a dome may be accompanied by violent outbursts of gas-rich magma for weeks, months or years.

The classical view of explosive eruptions may be summarized by a beer-bottle analogy. Like a carbonated beverage, magma contains gases that come out of solution under reduced pressure. Depending on the amount of pressure build-up in the bottle before removal of the cap, the frothy liquid may either blow itself apart and shoot out the top, or it may well out quietly and spill down the sides. The 'eruption' stops when the loss of gas brings the internal pressure down to the ambient level. In the geological analogue, explosive eruptions give way to more quiet lava flows and domes after the magma chamber loses a critical amount of gas. According to this interpretation, it is the distribution and quantity of vapour in the source magma body that controls the violence of the eruption and determines when explosive activity will end.

Jaupart and Allègre prefer a pressure-cooker model to explain transitions from explosive to passive eruptions. According to their idea, the pressurized vessel consists of both the sub-surface reservoir

and the overlying column of magma within the volcano. The pressure in the chamber and the quantity of gas in the magma both control the amount of pressure this molten rock feels. The key element of this model is that the fractured walls of the conduit act like a valve, bleeding off gas from the rising magma and promoting a shift from violent to quiescent eruptions. However,

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

A cloud of ash and steam dominates the skyline at Clark Air Base three days after Mount Pinatubo started erupting on 9 July, after 600 years of lying dormant.

gas can escape only if magma rises slowly enough, and if there is sufficient permeability in the surrounding rocks.

The new calculations show that the rate of gas loss is extremely sensitive, not only to the ascent of velocity of the magma but also to the pressure difference between the chamber and the atmosphere. Consequently, when an eruption reaches the dome-building stage, additional explosions are still likely owing to small fluctuations in pressure. At Mount St Helens in 1980, the major explosive event of 18 May was followed by an outburst a week later, and others in June, July and August. After each of these violent phases, a small dome appeared in the vent, only to be destroyed by the next blast.

The idea that permeability of host rocks can dictate the vigour of an explosive eruption was first promoted by John Eichelberger and his colleagues^{2,3}, on the basis of their studies of drill cores from some very young lava flows in California and New Mexico. But, because they lacked the analytical rigour of Jaupart and Allègre, these earlier workers made assumptions that were inconsistent with a range of field observations. Their papers focused on the extrusions that followed the explosive phases, and

claimed that all such domes emerged as highly inflated, permeable foams that collapsed to form dense lava as they travelled from the vent. Yet active domes like Mount St Helens have shown unambiguously that lava can erupt in a dense state that then inflates to bubble-rich pumice, rather than vice versa⁴.

A related incorrect assumption of these earlier studies is that lava emerges immediately after the pyroclastic activity ends, so that the gas-loss needed to cause a change in the eruptive behaviour must take place very rapidly. Yet, repose intervals of days, weeks and even decades may separate the end of explosive activity from the first appearance of lava. During such a hiatus, copious amounts of gas can be lost passively through the vent to the atmosphere, thus reducing the need for bleeding of gas into the country rock.

Although these distinctions may seem purely academic, they have important implications for late-stage explosive processes. If all gas must be lost from magma before it can form a lava dome, then there is no possibility that volatiles trapped within an advancing flow may decompress explosively. However, if extrusions can inflate after emergence, they retain the potential to effervesce violently if their

flow fronts collapse. Such behaviour has been documented at many volcanoes⁵, and may have caused the recent loss of life at Unzen. These 'endogenic' explosive processes represent one of the few important effects beyond the scope of Jaupart and Allègre's model.

The principal debate highlighted by this paper may be posed as one of 'nature versus nurture': do magmas completely control their eruptive destiny, or is their behaviour regulated fully by their environment? Jaupart and Allègre's model allows for both types of influence. Pressure variations in the magma body are the ultimate cause of shifts between explosive and extrusive activity, but loss of gas through permeable host-rocks may strongly affect such transitions. □

Jonathan Fink is in the Department of Geology, Arizona State University, Tempe, Arizona 85287, USA.

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A transcriptional tryst

Susanne Wagner and Michael R. Green

THERE has been a burst of activity centred upon the retinoblastoma gene and its protein product. The picture that is emerging from a spate of reports¹⁻⁸, including those on pages 249 and 251 of this issue^{6,7}, is still fuzzy. But it seems we may be witnessing the unification of three central areas of modern biology — cellular transformation, cell-cycle regulation, and transcription.

Oncoproteins

Three years or so ago, studies with DNA tumour viruses led to the discovery that several nuclear oncoproteins — including adenovirus E1a (ref. 9), simian virus 40 large T antigen (ref. 10) and papillomavirus E7 (ref. 11) — interact with Rb, the protein product of the retinoblastoma susceptibility gene, an anti-oncogene or tumour suppressor gene. Significantly, the viral oncoprotein regions that mediate binding to Rb overlapped with those essential for transformation. The conclusion of these findings was that DNA tumour virus oncoproteins transform cells by binding to and inactivating cellular Rb. Although appealing, this model was incomplete without an understanding of the molecular mechanism(s) by which Rb restrains cellular proliferation — in the absence of such knowledge, a pathway leading from Rb inactivation to cellular transformation could not be drawn. This deficiency has been at least partly overcome by the flurry of recent studies¹⁻⁸.

Although its precise function was unknown, several properties of Rb suggested that it has a role in transcriptional control and cell-cycle regulation. For example, it is a nuclear phosphoprotein¹² and bears amino-acid signatures reminiscent of DNA-binding proteins¹³; indeed, Rb can be shown to bind DNA¹².

Involvement in cell-cycle regulation was implied by the changes in Rb phosphorylation during progression of the cell cycle¹⁴⁻¹⁶. The master cell-cycle kinase, p34^{cdc2}, seems to be responsible, at least in part, for phosphorylating it¹⁷, and apparently it is underphosphorylated Rb which is active: this form is predominant in quiescent (G0) cells, and is the one bound by the DNA tumour virus oncoproteins^{18,19}.

To gain a foothold on Rb function, several groups have sought to identify the cellular protein(s) with which it interacts. Protein-affinity chromatography experiments reveal, somewhat surprisingly, that Rb binds to at least seven proteins found in the nucleus, which range in relative molecular mass from 26,000 to 150,000 (refs 20,21). Binding is

blocked by a peptide bearing a transforming region of a DNA tumour virus oncoprotein, implying that all seven cellular proteins interact with a common ('pocket') region of Rb.

In the new studies several of these cellular proteins have been identified, confirming that Rb is indeed involved in transcription. A cellular transcription factor called E2F (ref. 22) or DRTF (ref. 23) has been previously found to be associated with one or more cellular proteins, and three lines of evidence now indicate that E2F/DRTF interacts directly or indirectly with Rb — first, DNA-protein complexes formed on an E2F/DRTF site contain Rb (refs 1,2); second, Rb is present in a purified E2F preparation²; and third, the eluate of an Rb protein-affinity column contains E2F DNA-binding activity⁴.

The finding that E2F interacts with Rb helps explain several observations. E2F was originally identified through studies on transcription of the adenovirus E2 promoter, which contains two E2F binding sites. E1a activates E2 transcription in part by increasing E2F DNA-binding activity²⁴. One of several mechanisms by which E1a effects this increase is to release E2F from complexes with other cellular proteins²². This E2F-releasing activity is dependent upon the same regions of E1a that mediate binding to Rb (ref. 1). The inference is that E1a dissociates E2F from inactive complexes by binding to Rb.

Why do normal cells sequester E2F in an inactive form? E2F binding sites are present in the promoters of several cellular proliferation related genes^{4,25}. Expression of these genes can thus be regulated by sequestering one of their essential transcription factors.

In the absence of adenovirus infection, the cell must have a normal mechanism to release E2F from these inactive complexes. Several reports now show that such release is regulated by the cell cycle (see figure for a summary).

Using DNA mobility-shift assays, two E2F-containing complexes have been identified and found to be subject to cell-cycle regulation. One contains E2F and Rb, the other E2F and cyclin A (refs 2,5). A further complex containing E2F/DRTF, Rb and cyclin A has also been reported⁶. The E2F/Rb complex is found in G1 and disappears at the G1/S boundary, concomitant with phosphorylation of Rb. The

E2F/cyclin A complex is restricted to S phase, coinciding with the appearance of cyclin A (ref. 26). Both the E2F/Rb and E2F/cyclin A complex are dissociated by E1a, a result which fits in well with the finding that E1a also interacts directly with cyclin A (ref. 26). The interaction between E1a and cyclin A, like that between E1a and Rb, facilitates release of E2F.

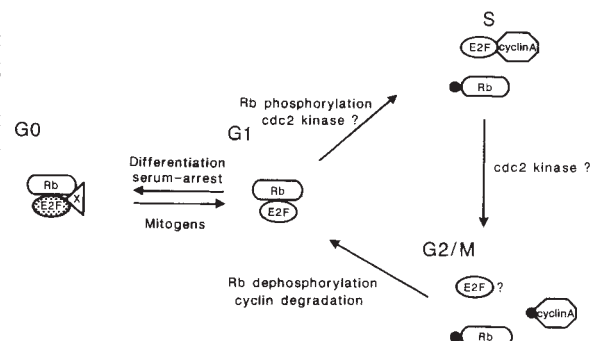
Although the E2F-containing complexes change during the cell cycle, total E2F DNA-binding activity remains relatively constant. But when cells are forced into quiescence, for example by serum deprivation, little or no E2F DNA-binding activity is detected². This implies the existence of an additional E2F-containing complex which, unlike the E2F/Rb and E2F/cyclin A complexes, cannot bind DNA. Mitogenic stimulation somehow dissociates E2F from this hypothetical complex.

Of particular interest is the putative component (X) of the hypothetical complex that prevents E2F DNA binding (see figure). Bagchi *et al.*³ appear to be on the trail of this entity. These investigators have partially purified an activity that inhibits E2F DNA binding and find that their fraction contains Rb along with several other proteins.

Immediate-early genes

But Rb's involvement in transcription does not end with E2F. Exposure of quiescent cells to mitogens activates transcription of so-called immediate-early genes, such as *c-fos* and *c-myc*. Transcriptional activation of these genes does not require *de novo* protein synthesis and therefore results from modification of pre-existing factors²⁷. Among the new results are some showing that Rb regulates the activity of both *c-fos* and *c-myc*.

Transcription of *c-fos* can be repressed by Rb in a transient expression assay²⁸. The *c-fos* promoter element responsible for Rb-mediated repression, termed RCE (Rb control element), contains sequence similarities to an E2F site. In the light of these results, an obvious inference is that Rb represses *c-fos* trans-



A speculative model of E2F complex formation during the cell cycle. The black circles denote phosphate groups. X is the putative component that prevents E2F DNA binding.

cription by sequestering E2F.

Whereas Rb regulates transcription of *c-fos*, regulation of *c-myc* occurs both transcriptionally and more directly. Like several other genes associated with cell proliferation, the *c-myc* promoter contains E2F-binding sites^{4,25}. This promoter region is involved in Rb-mediated repression of *c-myc* (ref. 29). But, in addition, protein-affinity chromatography reveals that Rb and the *c-myc* protein interact directly⁸. Significantly, this interaction involves the same Rb 'pocket' that mediates binding to viral oncoproteins. The region of the *c-myc* protein involved is the first 204 amino acids of the N terminus, which is a weak transcriptional activation domain and contributes to transformation (for review see ref. 30).

Taken together, these results establish a clear pattern: Rb restricts cellular growth by sequestering a variety of nuclear proteins involved in cellular proliferation. DNA tumour virus oncoproteins transform cells, at least in part, by releasing these cellular proteins from inactive complexes. The two cellular proteins that have been identified so far, E2F and the *c-myc* protein, are transcription factors. Whether all of the other cellular proteins that interact with immobilized Rb are also transcription factors, or have

some other nuclear function, remains to be seen. But it can be taken as an article of faith that they have an important part in cellular growth. With this conviction in mind, Defeo-Jones *et al.*⁷ now report the 'blind' cloning of two cellular proteins that bind to Rb. If we can be certain of anything, it is that deciphering the function of these and other Rb-

SOLAR SYSTEM

A plurality of worlds

S. J. Weidenschilling

THE most distant bodies visible in the Solar System are slowly yielding their secrets. The discovery of Pluto's large satellite, Charon, and observations of their mutual occultations led to the first meaningful measurements of their size and mass¹. The encounter of Voyager II with Neptune yielded a comparable understanding of its largest satellite, Triton (see figure). Surprisingly, Pluto and Triton turned out to be unexpectedly similar in size and density — (and hence, composition). S. A. Stern now argues² that these similarities are not coincidental; rather, Pluto and Triton are typical examples of large planetesimals that were once abundant in the outer Solar System. Most of these bodies (which may be conveniently called 'plutons') either collided and merged with the giant planets or were ejected from their vicinity by gravitational perturbations. Two were preserved owing to special circumstances — Pluto is in an orbital resonance with Neptune, and Triton was captured as a satellite of that planet. The original number of plutons must have been larger. Stern attempts to answer the questions: how many were there, and what became of them? His results suggest that there may well be others awaiting discovery.

Stern gives several lines of reasoning, none conclusive in itself, but contributing to a consistent picture. The axes of Uranus and Neptune are tilted far from their orbital axes, suggesting that they were each struck by large bodies, of the order of the Earth's mass³, while they formed through the accretion of smaller planetesimals. The existence of a few tens of such bodies with an assumed power-law size distribution would imply 1,000 – 10,000 plutons existed. This number is also consistent with an extrapolation to larger sizes of the estimated comet population⁴ in the hypothetical Oort cloud, on the outskirts of the Solar System.

Pluto and its outsized satellite form a seemingly unique 'binary planet'. Stern argues that this configuration actually must have been fairly common in the early Solar System; the probability that a

binding proteins will sharpen our insights into transcription, cell-cycle control and oncogenesis. □

Susanne Wagner and Michael R. Green are in the Program in Molecular Medicine, University of Massachusetts Medical Center, 373 Plantation Street, Worcester, Massachusetts 01605, USA.

truly unique body (or pair of bodies) would be preserved is miniscule. The only plausible way to make such a binary appears to involve an oblique collision between two bodies of comparable size⁵. The probability of such a collision depends on the number density of such objects, their relative velocities and the time available. If they filled a volume defined by Pluto's present orbital eccentricity and inclination, some 1,000 – 100,000 plutons would be needed for a typical body to experience a collision during the age of the Solar System. Although the actual relative velocities and the relevant volume probably were smaller, the available time for the collision was also almost certainly much less.

Triton's retrograde orbit clearly indicates that it was captured by Neptune. Various mechanisms have been proposed, including that it collided with a satellite already orbiting Neptune, a ring system⁶ or a circumplanetary nebula⁷. Stern favours a grazing encounter with Neptune's atmosphere. The appropriate cross-section to consider is that which allows Triton to be decelerated enough for it to be captured without being accreted onto the planet. Some 100 – 1,000 plutons are needed to produce one such encounter within the allotted time, assumed to be 500 million years. The capture efficiency, of the order of 1 per cent, implies 100 of the hypothetical plutons would be accreted by Neptune during this time.

One can argue with most of the assumptions. Over what volume of space were plutons distributed? What were their relative velocities? How much time elapsed before they were removed? What degree of improbability for the Pluto-Charon binary is acceptable? Surprisingly, Stern does not attempt to estimate the probability of Pluto's capture into orbital resonance with Neptune, which could provide an additional constraint.

Any such estimates are likely to have an elasticity of at least an order of magnitude. Still, it is scarcely credible that Pluto and Triton were the only such bodies in the early Solar System. The

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IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Neptune and its moon, Triton, in one of Voyager II's last pictures of the Solar System.

lower range of Stern's estimates does not conflict with current cosmogonical models: 1,000 plutons scarcely make three times Earth's mass. If these were accompanied by bodies as massive as Earth, the total mass would be comparable to the combined masses of Uranus and Neptune. But the higher estimates, of 10,000 or even 100,000 plutons, would imply amounts of mass and angular momentum that would be hard to reconcile with current models of planetary formation. One caveat should be kept in mind: the approach used to estimate the number of plutons — using relative velocities and collisional cross-sections — fails to explain the formation timescales of the outer planets, predicting that Uranus and Neptune would require more than the present age of the Solar System to accrete. Although this problem has long been recognized³, there is as yet no satisfactory solution. Presumably, some essential physics is missing from the cosmogonical models.

Although theory may be uncertain, there is still hope that observations can provide a definitive test. Where have the primordial plutons gone? Those that escaped collisions with the giant planets were removed from the region by gravitational scattering, sharing the same fate as the much smaller bodies that are comets. Some 10–50 per cent of these were scattered into distant orbits in the Oort cloud, some 10,000 astronomical units (AU) from the Sun (that is, 200 times further out than Pluto). There is unfortunately no way to detect objects at such distances. If the Oort cloud contains a thousand plutons, we may expect perturbations due to a nearby star to send a 1,000-km sized 'comet' into the

planetary system only once every 10 million years or so. Fortunately, we need not wait so long to see one. A consensus has developed that most of the short-period comets which we see in the inner Solar System are derived not from the Oort cloud, but from the Kuiper disk, a region beyond Neptune (30 AU) containing a population of comets having low orbital inclinations and moderate eccentricities⁸. In its original concept, the Kuiper disk was populated by bodies that formed *in situ* in the outer fringes of the solar nebula, where the density was too low to form planets. Recently, Torbett and Smoluchowski⁹ showed that orbits in this region with perihelia less than about 45 AU (a little beyond Pluto) are chaotic, and diffuse under the influence of perturbations by the giant planets.

Their conclusions have been strengthened by Levison¹⁰, who used a mapping technique to model this diffusion over times comparable with the age of the Solar System, and shows that objects formed near Neptune's orbit can reach distances exceeding 100 AU. Thus, the Kuiper disk should be populated, at least

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RESUME

Count down

THERE are between 1.84 and 2.57 million species of insect on Earth, according to new estimates that differ markedly from claims that there are upwards of 10 million of them. These earlier estimates were based on extrapolations from the numbers of beetle species (Order Coleoptera) found on 19 specimens of a single tropical tree species, and are highly sensitive to assumptions about host-plant specificity. Fewer problems beset the sampling of an area of Sulawesi, Indonesia, reported by I. D. Hodkinson and C. Casson (*Biol. J. Linn. Soc.* **43**, 101–109; 1991): strategies ranging from collection by hand to canopy fumigation netted 1,690 species of bug (Order Hemiptera) over a one-year period, 1,056 of which were previously unknown. That the decline in new species collected as the year progressed lends reliability to the final total — as well as the extrapolated estimates of global insect species numbers.

That's entertainment

Too much television is bad for you, they say, but in a remarkable case described by V. Ramani in *The New England Journal of Medicine* (**325**, 134–135; 1991), just one presenter causes problems. A 45-year-old woman suffered epileptic seizures triggered solely by the voice of Mary Hart, one of the hosts of the popular US TV programme *Entertainment Tonight*. The seizures were not triggered by background music or visual stimuli from the programme, different shows with similar formats, or other female voices. The patient's prognosis improved following drug treatment for blackout spells, and a strict avoidance of the show.

Slimier slime

THE hagfish, or slime eel, when annoyed, discharges a cloud of mucus that can engulf the author of its displeasure. It achieves this through the lysis of gland cells, each of which throws out a single fibre, some 3 µm thick and 60 cm long, made up largely of intermediate filaments (cytokeratins). E. A. Koch *et al.* (*J. struct. Biol.* **106**, 205–210; 1991) have now shown that these threads, with their parallel filament arrays, will further assemble into thick cable-like structures of striking appearance, very like those seen between hagfish eggs and responsible apparently for joining them into strings, like sausages. Koch *et al.* speculate that the mucus may tether the eggs to the spawning site, and note that the intimate details of the hagfish's sexual and reproductive mores are of some commercial interest — there are those who would like to breed the creatures, which are prized for the high quality of their 'eel-skin'.

in part, by bodies that formed closer to the Sun; one need not assume that the solar nebula extended to such a distance. The efficiency of placing such bodies into the disk is uncertain, but Stern estimates that of the order of 1 per cent of the original plutons may reside there; most would be beyond 50 AU.

Observational limits are not too restrictive. Tombaugh's new search¹¹ was essentially complete for plutons within 50 AU. Searches to fainter limits necessarily cover smaller areas. The most stringent limit implies an upper limit of about ten objects within 80 AU, unless

they are extremely dark¹². Deeper but less extensive searches formally allow up to 20,000 plutons to hide between 80 and 140 AU, but surely such a population would be accompanied by a significant number at smaller distances. A search that can detect bodies out to 100 AU will have a good chance of finding one or more objects. If none are out there, we will need greater ingenuity to explain the existence of Pluto and Triton. □

S. J. Weidenschilling is at the Planetary Science Institute, Tucson, Arizona 85719, USA.

EVOLUTION

Matriarchal liberation

John C. Avise

THE rules of molecular evolution seem made to be broken, and no piece of genetic material has shattered more evolutionary principles than has mitochondrial (mt) DNA¹. Do genes within an organismal lineage share a single evolutionary history? Not necessarily, as exemplified first and most forcefully by the disclosure that mtDNA is endosymbiotic in origin². Is the genetic code universal? No, as first demonstrated with the discovery of modified codon assignments in mammalian mtDNA³. Do functionally constrained sequences evolve slowly? Not invariably, as indicated by the rapid pace of nucleotide substitution in the functionally conservative animal mtDNA⁴ (probably due in part to inefficient DNA repair mechanisms). Do all genes in higher animals obey mendelian laws of segregation and independent assortment during sexual reproduction? No, as exemplified by the fact that mtDNA exhibits a strict maternal transmission.

Or does it? On page 255 of this issue⁵, Gyllenstein *et al.* report the detection of paternally derived mtDNA sequences in experimental backcross hybrids between the mice *Mus musculus* and *M. domesticus*. The proportion of paternal mtDNA within the heteroplasmic individuals was low (at most, one molecule in 1,000, the remainder being of maternal source), but the results nonetheless provide the first direct genetic evidence of effective leakage of paternal mtDNA in a vertebrate.

This finding comes on the heels of two other reports of departures from the axiom of complete maternal inheritance of mtDNA in higher animals. Kondo *et al.*⁶ used paternal-specific mtDNA probes in Southern blot experiments to reveal instances of paternal leakage in experimental populations of *Drosophila*; among 331 lines derived from hybrid progeny unidirectionally backcrossed to

genetically marked males, three strains (all from interspecific crosses) exhibited a complete replacement by paternal mtDNA within ten generations. Hoeh *et al.*⁷ used restriction digestion procedures to document the occurrence in mussels (*Mytilus edulis*) of heteroplasmic individuals carrying two highly distinct mtDNA genomes (sequence divergence $\approx$ 20 per cent). Such sequences are unlikely to have arisen by the accumulation of mutations within female lines, so the results were attributed to paternal input probably associated with recent interspecific hybridization.

Individual sperm carry small numbers of mtDNA molecules (about 50–100, compared to the 100,000 or more copies in a mature oocyte), and in some species they are known to penetrate the egg during fertilization (for discussion of this, and other aspects of mtDNA inheritance, see refs 1, 8 and 9). Earlier studies that employed less sensitive molecular assays may have failed to detect paternal input in hybrid progeny simply because of this numbers game. Another conventional speculation is that the usual maternal mode of inheritance may in some cases be strengthened by active oocyte involvement in the exclusion, degradation or under-replication of paternal mtDNA. Intriguingly, all three instances of paternal leakage found thus far involve inter- rather than intraspecific crosses. Gyllenstein *et al.*⁵ suggest that genetically distinct mtDNA from foreign sources might escape the exclusionary mechanisms normally aimed at closely related paternal molecules derived from fertilizations within species.

In any event, why all this fuss over low-level leakage of paternal mtDNA? The reason is that these discoveries raise evolutionary possibilities that previously were neglected, but now will require careful re-examination.

First, paternal leakage at least opens a

window of opportunity for physical recombination between mtDNA molecules from separate parents. Although such intermolecular recombination has not yet been discovered in higher animal mtDNA (in heteroplasmic *Mytilus*⁷, the distinct mtDNAs retained their separate identities), such a phenomenon could complicate reconstruction and interpretation of genealogies based on mtDNA. Second, because mtDNA numbers probably go through bottlenecks during oogenesis¹⁰, paternal mtDNA might occasionally be the 'lucky' survivor and take over a maternal lineage (as in the *Drosophila* study⁶). Such paternal invasions would provide a mtDNA genetic bridge between otherwise isolated cytoplasmic matrilineages. And, depending on frequency, they could have ramifications in such areas as the interpretation of mtDNA gene trees as estimates of strict matriarchal phylogeny; estimation of sex-specific demographic parameters, including effective female population size and gene flow⁶; the likelihood of occurrence of repetitive selfish elements within mtDNA (in principle, genes with selfish motives gain no fitness advantage by becoming repetitive within a uniparentally transmitted, non-recombining genome¹¹); and, finally, the degree to which potential conflicts of interest between nuclear and cytoplasmic genes stem from the contrasting evolutionary strategies expected for biparentally as opposed to uniparentally transmitted genomes^{1,11}.

As to the evolutionary significance, it remains to be seen whether leaks of paternal mtDNA are analogous to drips from a leaky tap (an inconsequential source of noise), or to seepage from cracks in a dike (perhaps a prelude to the bursting of yet another genetic principle, in this case one of mtDNA's own making). Given the history of mitochondrial incursions into conventional evolutionary doctrine, it may be wise to keep an eye on this trickle. □

John C. Avise is in the Department of Genetics, University of Georgia, Athens, Georgia 30602, USA.

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A marriage is consummated

Peter A. Lawrence and Andrew Tomlinson

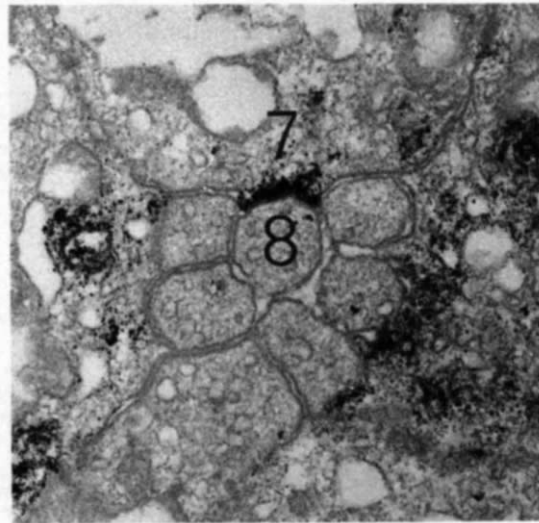
PICTURE a child's jigsaw puzzle — the kind with a few large pieces, but with a significant bit missing. The jigsaw analogy is apt for the insect compound eye, where not only do all the cells fit together intricately, but the pieces of understanding interlock in such a satisfying way that they leave precisely shaped holes that ask to be occupied. One such space is now filled by Krämer *et al.* (see page 207 of this issue¹), who have apparently found the ligand for the sevenless protein.

The *Drosophila* eye consists of hundreds of repeating units called ommatidia, and each one is made as a unit². Initially, one set of photoreceptor cells called R8 are selected from a sheet of epidermis². This process probably depends on lateral inhibition³ (the mechanism by which individual cells are selected from a population and, once selected, develop in a new direction and also inhibit nearby cells from doing the same)⁴. After an R8 cell is formed, the ommatidium builds outwards as adjoining epidermal cells are recruited in a stereotyped pattern, first R2 and R5, then R3, R4 and R1, R6, and finally R7 (refs 2,5). The other cells of the ommatidium, the cone cells that form the lens, the pigment cells that insulate each ommatidium from its neighbours and the founding cells of eye bristles, are all added on by further recruitment. When this sequence is complete, there are still a group of surplus unallocated cells; these die to leave the whole structure as perfect and as precise as a crystal⁶.

Because the eye is dispensable, mutations which damage it will not be lethal to the fly. So genes that effect specific steps in this process have been identified. For example, the *rough* gene is needed to specify cells R2 and R5. In its absence they do not develop properly and cannot trigger the events that would normally occur next⁷.

The recruitment of the R7 cell has been especially well investigated, beginning with the isolation of *sevenless*⁻ flies in 1976 (ref.8); these mutants have no R7 cells and the cell that would have made the R7 cell makes a cone cell instead⁹. Studies with genetic mosaics show that the *sevenless* gene is needed only in the R7 cell itself, so the requirement for the gene is cell autonomous⁸. The *sevenless* gene encodes a tyrosine-kinase receptor protein that is predicted to be in the membrane¹⁰. In the electron microscope, the sevenless antigen can be seen on the apical plasma membrane of the R7 cells, and seems to form patches where the R7 cell touches the founding

R8 cells¹¹. This is a striking visual image, one which suggests that there is a ligand on the R8 cell that binds to the sevenless protein molecules on the R7 cell and thereby creates and tethers rafts of re-



Developing photoreceptor cells in the *Drosophila* eye; sevenless antigen is seen where R7 contacts R8. Receptor protein (see figure).

Three years ago, Zipursky's group identified a gene that might encode this ligand and called it *bride of sevenless* (*boss*)¹². Mutations in *boss* cause a phenotype identical to that caused by mutations in *sevenless*, but analysis of eyes that are a mixture of *boss*⁺ and *boss*⁻ cells show that the gene is not needed in the R7 cell itself. The requirement for *boss* is therefore not cell autonomous. Instead, the phenotype of each individual ommatidium correlates with the genotype of the R8 cell. If the R8 cell is *boss*⁺, the ommatidium will have the full complement of eight photoreceptor cells, if it is *boss*⁻ it will have seven, and these rules apply whatever the genotypes of the other photoreceptor cells. The genetic mosaics suggest that the *boss* gene product should be looked for in the R8 cells¹².

The *boss* gene encodes an integral membrane protein with seven transmembrane domains¹³, and Kramer *et al.*¹ now offer persuasive arguments that this protein is indeed the ligand for *sevenless*. First, using electron microscopy they show that the protein is located on the apical surfaces of the R8 cells. It is also found inside the R8 cells in smaller multivesicular bodies and, significantly, inside each R7 cell in an extraordinarily large membrane-bound body — so big it is easily seen in the light microscope. In the absence of the *sevenless* gene, the boss antigen is not found in the R7 cells.

Second, they show that the boss and sevenless proteins can interact; when they are expressed separately in transfected tissue culture cell lines the cells do not stick together, but when the two cell lines are mixed they form aggregates.

In *Drosophila* molecular genetics, it is commonplace for the ligand to be known but not the receptor (or vice versa).

Here is a welcome exception. Moreover this is an example of molecular transduction not between a diffusing ligand and a membrane-bound receptor, but, apparently, between two integral membrane proteins. If it is, how does the boss antigen enter the R7 cell? Further questions requiring answers are, for example, why does only the prospective R7 cell take up the boss protein — other cells that express the sevenless protein are in contact with R8, and yet do not appear to contain the antigen. Krämer *et al.* say that the uptake of the boss protein might be an important part of the signalling process. If this is so it cannot be essential, as

cells which contain artificially high amounts of sevenless protein can be self-activated and transform into R7 cells¹⁴ — moreover this self-activation can occur in the absence of the *boss* gene¹⁴. So, how does the boss protein actually produce the change in fate of the cell? And what is the huge 'multivesicular body' in the R7 cell?

The work discussed here is the latest in a line of imaginative and illuminating studies carried out by Zipursky's group. They illustrate how, increasingly, genetic analysis of pattern formation takes us from the relatively clear logic of genetics into the thickets of cell biology. □

Peter A. Lawrence and Andrew Tomlinson are in the MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

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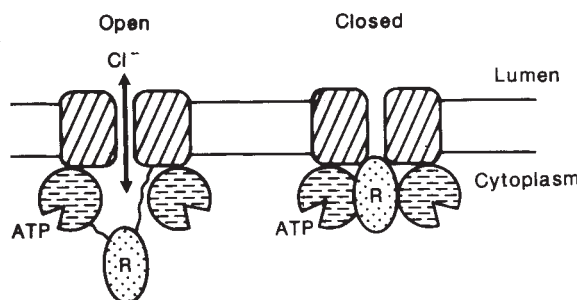
Channelling our thoughts

Christopher F. Higgins and Stephen C. Hyde

THE jury, it seems, is now in. After a period of hectic activity and a great deal of controversy, two papers in last week's *Science*^{1,2} by the groups led by Michael Welsh and Alan Smith provide compelling evidence that the protein encoded by the cystic fibrosis gene is a regulated chloride channel. Earlier this year, as discussed in News and Views³, it was shown that expression of the protein (known as CFTR) in several different cell types can generate a new cyclic AMP-regulated Cl^- channel^{4,5}. In an elegant series of experiments, Welsh, Smith and their collaborators have now introduced specific amino-acid changes into CFTR which alter the characteristics of this channel. The results effectively exclude the possibility that CFTR activates an endogenous channel by acting as the transporter of some regulatory molecule.

Cystic fibrosis transmembrane conductance regulator (to give CFTR its full name) consists of five domains⁶ (see figure). In the first of the two sets of experiments¹, positively charged amino acids in the transmembrane domains were, separately, replaced with negatively charged residues. This altered the ion selectivity of the channel in favour of I^-

rather than Cl^- . The simplest interpretation is that these charged residues contribute to a transmembrane 'pore' and play a direct part in determining ion selectivity. But the effects of these charge rever-



Model for CFTR function and regulation. Two transmembrane domains (hatched) form a pathway for Cl^- movement across the bilayer. The R domain opens and closes the channel in response to cAMP-activated protein kinases. The function of the ATP-binding domains (dashed lines) remains unclear.

sals on the ion selectivity are, perhaps, more subtle than might have been anticipated. An alternative interpretation is that the amino-acid changes influence ion selectivity indirectly, through protein-protein interactions (dimerization of CFTR, or an interaction between CFTR and another protein).

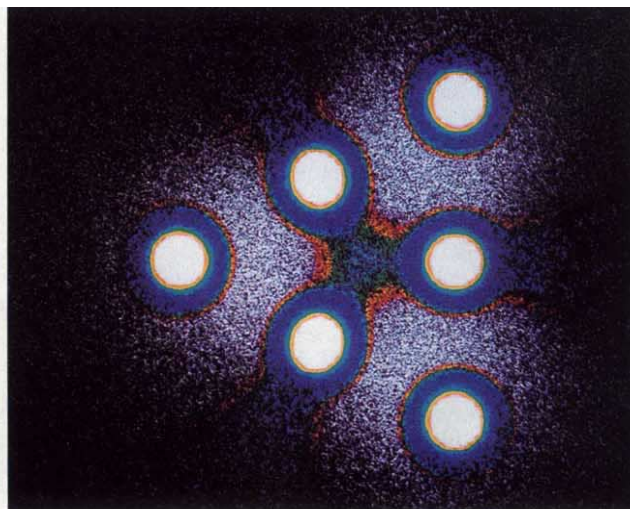
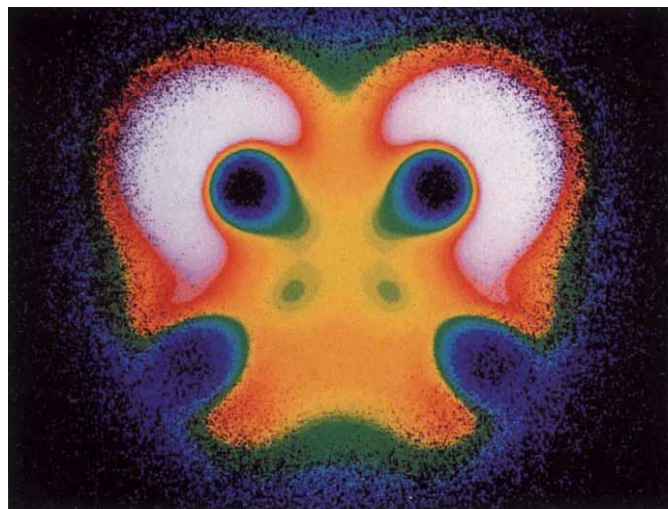
In the second piece of work², deletion of the R domain of CFTR was found to

leave the channel permanently open, implicating this domain in the opening and closing of the channel in response to cAMP-activated protein kinases (see figure). The observation that a mutation in one of the ATP-binding domains is suppressed by deletion of the R domain has been interpreted as showing that the ATP-binding domains may also have a regulatory role²: perhaps the R domain is moved into and out of the channel in an ATP-dependent fashion. In total, these data are convincing and strongly support the notion that CFTR is itself a regulated Cl^- channel or, at the very least, a component of a multisubunit channel.

Several riders must be added to this conclusion. First, the characteristics of the CFTR channel are very different from those of the voltage-gated, outwardly rectifying channel which has been associated with cystic fibrosis in the past; indeed, it now seems that the outward-rectifying channel may have little to do with cystic fibrosis⁷. Second, it remains to be shown that a channel with the characteristics of that conferred by CFTR is defective in cystic fibrosis cells; a similar channel has, however, been measured in normal epithelia^{1,7}. Finally, most mutations to the cystic fibrosis gene alter the ATP-binding domains of CFTR, yet the function of these domains is far from clear.

Besides their obvious importance for cystic fibrosis research, the new results

Electron density maps with a difference



Within every chemist, it seems, there lurks a Salvador Dali. What brings this repressed artistic bent out into the open is the need to depict some unusual trait of a complex molecule. A. Savin *et al.* (*Angew. Chemie Int. Ed. Engl.* **30**, 409–412; 1991) have turned their attention to the old problem of visualizing the distribution of electrons in molecular bonds. To emphasize the signifi-

cance of localization of electrons in bonding, they have resorted to the electron-localization function, a mathematical measure, which they have colour-coded, of the probability of finding two electrons in the vicinity of one another. The two molecules shown here are (left) a slice through Cu_4Sn_4 in the heterocubane structure (made of intersecting tetrahedra of tin and

copper) and a planar Li_6 cluster. Regions of highest density (the distorted lone pairs of tin in Sn_4Cu_4 and stable three-centre bonds in Li_6) are white. Density decreases through brown and blue to black. The tin nuclei appear black owing to the calculational method. The separation between the core and valence electrons of lithium is also apparent. (Courtesy A. Savin.)

challenge current thinking about channels and transporters. CFTR is unlike any known channel but, in sequence and domain organization, seems to be a typical member of the ABC (ATP-binding cassette) superfamily of transporters⁸. Over 40 ABC transporters have so far been identified, and all those which have been sufficiently well characterized (including the human multidrug resistance protein and numerous bacterial 'periplasmic' transport systems) are, unambiguously, ATP-dependent active transporters. It is important to emphasize here that the CFTR-dependent movement of Cl^- across the membrane is channel-like, and that the available data are not consistent with the idea that CFTR is an ATP-dependent Cl^- transporter.

At least as they are defined experimentally, empirical distinctions are generally made between channels and transporters. In essence, transporters are 'enzyme-like', interacting stoichiometrically with their substrate and undergoing a kinetically defined conformational change during each transport cycle (sometimes considered as a reorientation of the substrate-recognition site between the two faces of the membrane). In contrast, channels may be more akin to 'pores' which, when open, allow free and non-stoichiometric passage to molecules with appropriate characteristics. One consequence of these differences is that the turnover number (the number of molecules of substrate crossing the membrane each second) of a transporter is limited. Channels, in contrast, are ultimately diffusion limited and frequently have turnover numbers several orders of magnitude greater than that of any transporter. Additionally, until now perhaps, the sequence, structure and organization of known channels and transporters has appeared to be very different⁹.

We have, then, a conundrum — CFTR 'looks like' a transporter yet functions as a channel. One way out of this is to propose that CFTR is multifunctional, that it is both a Cl^- channel and a transporter for an unidentified substrate. In this context it may be relevant that the putative protein exporter of the endoplasmic reticulum forms an ion channel when deprived of its normal substrate¹⁰. Perhaps monomeric CFTR is a transporter, and a Cl^- channel is generated when it associates as a homo-

or heterodimer.

Alternatively, Occam's razor directs us to the simpler supposition that the only function of CFTR is its Cl^- channel activity. As there is no doubt that other ABC proteins are transporters, the provocative implication is that, despite the kinetic and other differences between channels and transporters, the actual pathway which a substrate takes across the lipid bilayer may not be very different. This limits the types of models that can be envisaged; channels may tell us

something about transporters and vice versa. Working out the structure of the transmembrane pathway of channels and transporters, and the molecular mechanisms by which solute is translocated across a lipid bilayer, will be one of the major tasks of the next few years. □

Christopher F. Higgins and Stephen C. Hyde are in the Imperial Cancer Research Fund Laboratories, Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK.

GLOBULAR CLUSTERS

New tools of the trade

Charles Bailyn

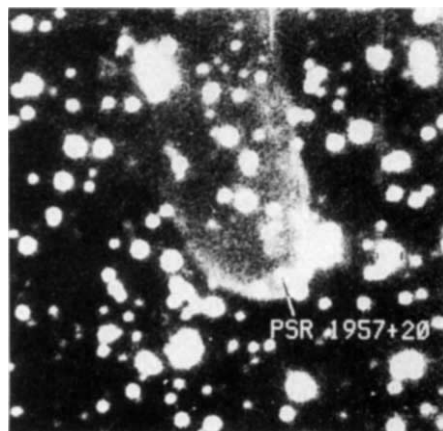
SINCE the first millisecond pulsar in a globular star cluster was reported¹ in 1987, the trickle of discoveries of these objects (a few each year in 1987 and 1988) has become a torrent. On page 219 of this issue², Manchester *et al.* report the discovery of ten new pulsars in a single cluster. Although this unexpectedly large pool of pulsars has, if anything, deepened the mystery surrounding their origin, workers in other fields of astrophysics are finding them to be useful tools for addressing problems in cluster dynamics and galactic structure³⁻⁵.

The ten millisecond pulsars Manchester *et al.* have found in the cluster 47 Tucanae exacerbate a problem which has created some contention recently: the birthrate of millisecond pulsars (stellar radio beacons that spin at rates of up to 600 times a second), both in clusters and elsewhere in our Galaxy, appears to be substantially greater than that of the X-ray emitting binary stars which are their supposed precursors^{6,7}. Attempted resolutions of this difficulty include drastically altered models of the evolution of X-ray binary stars⁸, alternative classes of precursor objects⁶, and clever accounting schemes that purport to demonstrate that the numbers of pulsars may still be compatible with old ideas concerning their origin⁹.

The new discoveries make the last suggestion difficult to sustain: a recent study which concluded that the 150 or so galactic globular clusters contain at most 300 – 700 pulsars⁹ (a number which would be compatible with standard theories of pulsar birth) predicts no more than a total of three to five pulsars in 47 Tucanae. But there are doubtless many more than the eleven discovered so far: globular clusters are so far away that many of the pulsars they contain should be invisible. Indeed several of the pulsars detected by Manchester *et al.* were found only because of the occasional amplifying effects of interstellar scintillation. As the cluster continues to be

observed, therefore, we can confidently expect many more pulsars to be discovered in it.

But the new pulsars are interesting not only for their own sake, but also as probes of and influences on their surroundings. On page 221 of this issue³, for example, Spiegel points out that the radiation field generated by numerous pulsars might explain the curious abs-



The bow shock around the eclipsing millisecond pulsar PSR 1957+20, demonstrating the influence of the pulsar's radiation field on surrounding gas¹¹.

ence of gas and dust in globular clusters. As stars evolve, they shed much of their outer layers; some of this stellar debris can then coalesce to make new stars. But observations so far have revealed the presence of gas in only the most tightly bound of the globular clusters¹⁰, and there is no second generation of stars to be found. The radiation field from single pulsars has been shown to push gas away from the pulsar (see figure) and Spiegel shows that a few dozen pulsars would be enough to sweep a cluster clean.

Pulsars can be used to detect gas, as well as remove it. This is because ionized gas between the pulsar and observer delays the arrival of the pulses, with lower frequencies being delayed more.

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The change in pulse arrival time with frequency therefore determines the amount of gas along the line of sight. Because the distances to globular clusters are known by other means, this effect can be used to map the distribution of ionized gas in the Galaxy⁴. Initial results have confirmed earlier evidence that a layer of ionized gas over a kiloparsec thick blankets the galactic disk (for a sense of scale, the Sun is 8 kiloparsecs from the Galactic Centre); as more clusters are found to contain pulsars, more detailed studies of the distribution of this gas will be possible.

The exquisite accuracy possible in timing millisecond pulsars means that they can also be used as mass detectors. The gravitational acceleration of a pulsar along the line of sight due to the material surrounding it results in changes in the pulse period and, for binary pulsars, the binary orbital period as well. In the deep gravitational well of a globular cluster, these changes are measurable, and significant compared with the intrinsic rates of change. Thus measurements of the pulse and binary period derivatives can be used to constrain the dis-

tribution of visible and invisible mass in a cluster. A recent analysis by Phinney⁵ of the pulsars in the globular cluster M15, has determined that the core of that cluster must contain thousands of dim, heavy objects. This may be the pool of neutron stars required to create the pulsars currently being discovered. A similar analysis should shortly become possible in 47 Tucanae. The comparison between these two dense clusters, which differ in metallicity and dynamical state, will be especially interesting. □

Charles Bailyn is in the Department of Astronomy, Yale University, PO Box 6666, New Haven, Connecticut 06511, USA.

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CLIMATE CHANGE

Credit the oceans?

Curt Covey

ONE of the biggest unknowns in understanding climate change is the role of the oceans. Calculations by Rind and Chandler now show¹ how important this may be: the major climate changes of the past 180 million years, they suggest, can be understood entirely in terms of changes in ocean circulation.

Nearly two decades have passed since the first satellite measurements of Earth's radiation budget suggested that the oceans carry much if not most of the burden of transporting heat from the planet's warm equatorial regions toward the poles². Palaeoclimatologists have since attributed a number of past changes in climate to variations of oceanic heat transport³. And with recent concerns about a greenhouse effect amplified by human activities has come the fear that future climatic changes might be worsened by "unpleasant surprises" in the form of relatively sudden reorganizations of ocean circulation⁴.

Speculation has outdistanced calculation for a simple reason⁵: although the atmosphere and oceans characteristically transport the same amount of mass per unit time, the oceans have nearly 400 times more mass and take a correspondingly longer time to adjust to changes. A few hours on a supercomputer suffices for an atmospheric general circulation model (GCM) to shift from one climatic equi-

brium state to another, given boundary conditions in the form of constant sea surface temperatures. Difficulties for the analogous exercise with an oceanic GCM can be imagined (and would become worse if it were necessary to explicitly represent so-called mesoscale eddies, the oceans' equivalent of weather systems). Now, however, Rind and Chandler¹ have cleverly employed an atmospheric GCM to approach a subset of the problem without the need for time-consuming

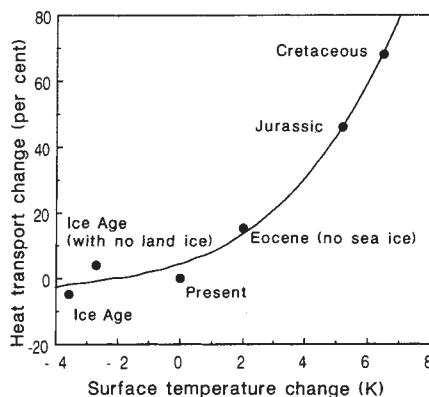
oceanic simulation. The techniques are not new, but they are milked for nearly all they are worth, producing a survey of potential connections between oceanic heat transport and climate, as seen by models, from the Jurassic (180 million years ago) through to the late twenty-first century.

How can one infer ocean/climate connections from calculations that fix oceanic behaviour at the start? The key is to seek only two quantities: the oceanic heat transport necessary to sustain a given climate; and the effect upon the climate of a given oceanic heat transport. The first quantity is obtained by fixing sea surface temperatures at whatever values seem to fit a past or potential future climate, then letting the atmospheric GCM run to equilibrium. Annual mean fluxes of energy into and out of the sea surface, automatically calculated in the course of the run, give the divergence or convergence of implied oceanic heat transport.

Rind and Chandler's control experiment employing observed present-day sea surface temperatures gives plausible values for today's oceanic heat transport. The authors also calculated the change in heat transport required for five different climates: the warm Jurassic and even warmer Cretaceous; the Eocene, when the tropics were no warmer than today but the poles were virtually free of sea ice; the peak of the last Ice Age 18,000 years ago; and Ice Age sea surface temperatures without land ice (perhaps representing the beginning of an Ice Age). Globally averaged, the temperature changes from the present and the changes in oceanic heat transport display a striking correlation, as shown in the figure.

Rind and Chandler thus demonstrate that without altering greenhouse gases, the full range of temperatures experienced on Earth over the past 180 million years — albeit with the Cretaceous at the low end of its observational error bar — could have been generated by a factor-of-two variation of oceanic heat transport. (One caveat: the GCM the authors use is more sensitive than most, giving predictions on the high side of the canonical $3 \pm 1.5^\circ\text{C}$ expected for CO_2 doubling.)

The second quantity, response of climate to a given amount of oceanic heat transport, Rind and Chandler assess by altering the form of atmospheric GCM's lower boundary conditions to specify oceanic heat transport rather than surface temperature. As they point out, this study complements similar work by Stanley Thompson and me⁶; we vary oceanic heat transport outside high latitudes all the way from zero to twice our best guess at present-day values, but fix the amount of heat transport underneath sea ice at present-day levels. Rind and Chandler take the opposite tack: they use their first technique to determine how much ocea-



Change in oceanic heat transport required to produce a given change in globally averaged surface temperature, as computed from Rind and Chandler's atmospheric GCM (plotted from Rind and Chandler's Table 6). The best-fit cubic curve through the points is shown.

nic heat transport would be required to melt all sea ice, then use that elevated transport as input to their GCM. Because the two studies make assumptions at opposite ends of the spectrum of possibilities for sea-ice feedback, they evidently bracket the truth between them, but the gap is uncomfortably large. Rind and Chandler claim an ice-eradicating oceanic circulation is "not entirely unreasonable" and, although they shy away from estimating the volume transports implied, these bear out their claim: at 60° N latitude, oceanic heat flows poleward at the rate of 10^{15} watts (twice the estimated present-day value), and sea surface temperature increases to 10 °C, so that if bottom water remains at about 3 °C, the volume transport must be $3.4 \times 10^7 \text{ m}^3 \text{ s}^{-1}$, not an outrageous increase over Broecker's estimate of $2 \pm 0.5 \times 10^7 \text{ m}^3 \text{ s}^{-1}$ for present-day North Atlantic Deep Water formation⁷.

Rind and Chandler also note that "climate feedbacks which influence the wind-driven surface circulation and the deep water thermohaline circulation would appear to be in the right direction to sustain the increased [oceanic heat] transports". The feedbacks are straightforward. Expansion of subtropical maxima in surface atmospheric pressure leads through geostrophy to enhanced anticyclonic wind vorticity (in oceanographers' terms, negative windstress curl). In other words, the strength increases for both east winds at low latitudes and west winds at high latitudes, which would be expected to increase warm poleward boundary currents such as the Gulf Stream. Simultaneously the disappearance of sea ice leads to greater evaporation in polar seas, which would lead to greater surface salinity and presumably an increased density-driven overturning circulation involving warm poleward surface currents and cold equatorward deep currents. However, as Rind and Chandler point out, elucidation of "whether the magnitude of the ocean dynamical changes is appropriate will have to await the development of coupled atmosphere/ocean models".

Despite their radically different assumptions about sea ice, our work and that of Rind and Chandler share one important general conclusion: the climate resulting from enhanced oceanic heat transport is quite different from that resulting from the greenhouse effect, with much more warming at the poles and much less warming (even cooling, in our simulation) in the tropics. Although geological evidence such as from the Vostok ice cores shows greenhouse gas concentrations to be correlated with past global temperatures, the changing concentrations could be more an effect of altered ocean circulation than a primary cause of the temperature variations. If Earth's climate history was generated more by

variations of oceanic heat transport than by variations of atmospheric composition — a possibility I find difficult to ignore after reading Rind and Chandler's paper — then using direct analogy with past climates to estimate the global warming expected from the human contribution to greenhouse gases is a questionable exercise, and we must depend more than ever on computer models to forecast the climate of the next century. That is a disconcerting prospect given the notorious 'climate drift' of coupled oceanic-atmosphere GCMs (possibly connected to the difficulties with time scale discussed above)⁸.

What are the logical next steps in elucidating ocean/climate interactions? The long timescales of oceanic circulation can be accommodated by accelerated forcing to equilibrium⁹ or by filtering out irrelevant (we hope) 'fast dynamics'¹⁰. Thus equipped, oceanic GCMs can at least stand on their own and address the mirror image of the question that Rind and Chandler posed: given an atmospheric climate thought to represent Earth in the past, what is the oceanic circulation? (First results from this sort of exercise are disturbing: Barron and Peterson¹¹ find Cretaceous ocean circulation just the opposite of conventional expectations.) Certainly oceanographers interested in climate can formulate their GCMs' boundary conditions more cleverly than is traditionally done — with relaxation to observed surface temperature¹², which yields the curious property that when model-simulated sea surface temperatures agree precisely with observations, energy flux into or out of the oceans (and hence oceanic heat transport) is precisely zero. And although construction of validated, fully coupled models of the oceans atmosphere, biosphere and cryosphere is a daunting goal, the appropriate tool in the form of raw computing power, may soon be available with the arrival of massively parallel computers. Whatever the difficulty of the questions, the importance of the answers guarantees future attention to the oceans' impact on climate. □

Curt Covey is at Lawrence-Livermore National Laboratory, PO Box 808, L-264, Livermore, California 94550, USA.

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Automatic marks

EVERY academic year, students and examiners confront each other in the painful scholastic ritual of examinations. In scientific subjects, the questions at least have a right answer for the examiner to look for. But the marking of essays and appreciations, a crucial element of liberal education, is harder to automate. Daedalus is working on it.

He recalls the way in which psychologists measure personality. They draw up a list of several thousand silly questions, and put them to subjects whose personalities have been evaluated by the most informed and intensive methods. Analysis of the outcome usually shows that a few hundred of the resulting silly answers correlate highly with, and seem good predictors for, certain established aspects of the subjects' personalities. These predictive questions then form a 'multiphasic inventory' which can be applied to further subjects.

So Daedalus is trying this idea out on examination essays. Computer-analysis is already used for testing authorship, and simple indices like the 'fog-index' of writing, a measure of average word-length, are widely recognized. DREADCO educationalists are subjecting a set of traditionally-marked essays to thousands of similar computerized tests. The semicolon to comma ratio, the number of words used exactly twice, the balance between odd and even letters of the alphabet, the fractional occurrence of the word 'involved' — all are being determined. Most of these tests will show no predictive value. But a small proportion will be found to correlate with the assessed worth of the answer. Further answers can be marked purely by computer-evaluating these successful measures.

The resulting 'educational multiphasic inventories' will have to be kept secret. They will also need updating. Progressive and alert examining boards will periodically recalibrate their inventories against intensively-marked papers, just as a good factory regularly checks its shop-floor instruments in the standards laboratory. But the routine agony of conscientious marking, the struggle to understand what (if anything) the candidate is trying to say, will be eliminated.

Good riddance, says Daedalus. In his view, examinations don't measure understanding anyway. They measure 'grip': the ability to assess the best practical way of meeting a challenge. If the examinations were held under water, the same people would come top. They would be the ones who would work out the best way to answer an examination under water. They will respond to the new computerized challenge just as well.

DAVID JONES

Early cosmic background

SIR — The existence of a black-body radiation at 3 K in the cosmological background is one of the essential supports of the Big Bang model of the Universe. The first measurement of the 'noise' temperature associated with this radiation is attributed to Penzias and Wilson in their famous paper published in 1965 (ref. 1), in which they announced a 3.5 ± 1 K measurement at a wavelength of 7 cm using a detector cooled by liquid helium. In a companion paper, Dicke, Peebles, Roll and Wilkinson interpreted these results as a fossil Universe black-body radiation². Weinberg has wondered why this cosmological background was not discovered earlier³. We can give a partial answer to this question by calling attention to a PhD dissertation that has until now been overlooked.

As early as 1955, E. Le Roux measured the absolute temperature of the sky as 3 ± 2 K while working at the Laboratoire de Radioastronomie in the Ecole Normale Supérieure in Paris⁴, in agreement with the most recent measurements from satellites and rockets⁵, which give a value of 2.736 ± 0.017 K. The three measurements performed by Le Roux for three different elevation angles of the antenna in 1955 led to an arithmetic mean value equal to 2.8 K and allowed him to show that 3 ± 2 K is the only possible value to explain the consistency between the three measured values and the three corresponding equations. Moreover, the isotropy of this radiation is better than 0.5 K, as shown by the isophotes in ref. 6.

Le Roux attempted to compare the experimental spectrum obtained from some other available measurements with the black-body spectrum in the Rayleigh-Jeans approximation. Unfortunately, this comparison failed because of the erroneous values obtained in other papers. Nevertheless, he suggested the extragalactic origin of this radiation, although he did not compare it to early predictions of the cosmological background⁷.

Le Roux used an antenna which was part of a German radar system given to Professor Rocard by the British army after the Second World War. This antenna worked at 33 cm and its spatial detectivity was known with great precision. Hence, Le Roux knew quantitatively the contribution of every solid angle element to the antenna temperature. The detector carefully matched to the antenna was not cooled in this case and considerable noise, equivalent to a temperature of 1,450 K, is superimposed on the useful signal. To avoid too much uncertainty in the value of the 'sky temperature', Le Roux used a clever experimental method which we have described elsewhere⁸. He recorded the signals obtained when the axis of the antenna was swept from the zenith to an elevation angle of -3° below the horizon. At every moment, the antenna temperature is the result of the temperatures of both the sky and the ground, with exactly known coefficients

depending on the elevation angle of the antenna axis. Consequently, the antenna temperature variations from the zenith to the ground allowed Le Roux to derive the slope of the characteristics of the apparatus and to deduce several consistent measurements of the 'sky temperature'. This differential method allowed him to reach an accuracy comparable to that of Penzias and Wilson without having to cool the apparatus.

ALBERT LE FLOCH
FABIEN BRETENAKER*

Laboratoire d'Electronique Quantique-
Physique des Lasers,
Université de Rennes I,
Campus de Beaulieu,
35042 Rennes, Cedex, France

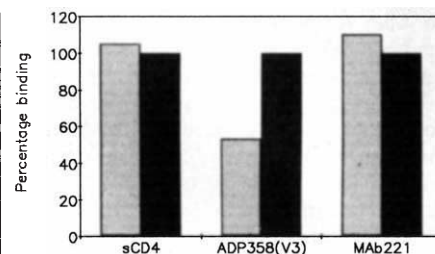
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*Also at Société d'Applications Générales d'Electricité et de Mécanique, 70-74 Rue de le Tour Billy, BP72, 95101 Argenteuil Cedex, France.

Anti-HIV drug mechanism

SIR — Amino acid derivatives such as *N*-(*n*-butyl)-deoxyjirimycin (BuDNJ), have shown exceptional promise *in vitro* as potential anti-HIV compounds¹⁻⁴. The primary mode of action of these compounds is the inhibition of α -glucosidases in the infected cell, leading to hyperglycosylation of the virion surface glycoprotein gp160 (refs 1,5,6). The exact biological consequences of hyperglycosylation and the link with the observed anti-viral activity remain uncertain. BuDNJ-treated HIV glycoproteins continue to bind to CD4 and, although the processing of gp160 to gp120 and gp41 required for viral infectivity is retarded, it is neither abolished nor reduced sufficiently wholly to explain the anti-viral effect of DNJ⁶. We have observed a conformational change in gp120 following its production in the presence of BuDNJ that could help to explain the drug's action.

We expressed gp120 using recombinant baculoviruses⁷ in the presence or absence of BuDNJ (0.5 mg ml⁻¹) and normalized for the amount of antigen present by quantitative western blot. The relative molecular mass of BuDNJ-treated gp120 was approximately 10,000 greater than the untreated fraction, in keeping with hyperglycosylation of the glycoprotein, but both fractions bound equally well to sCD4 and to a polyvalent antiserum against gp120 (S216), indicating no gross hyperglycosylation-induced confor-



Gp120 was expressed using recombinant baculoviruses as described⁷ with or without the addition of 0.5 mg ml⁻¹ BuDNJ. Antigen present in the supernatant of infected cells was tethered to a solid phase using the anti-gp120 serum D7324 (Aalto BioReagents, Dublin) and incubated with sCD4, ADP358 (an anti-V3 monoclonal antibody) or MAb 221 (a monoclonal antibody directed to the 44 amino acids at the carboxy terminus of gp120; ref. 10). Binding to gp120 was detected by incubation with the relevant conjugates (anti-CD4 or anti-mouse) and results are expressed as percentage of the binding observed with the BuDNJ-treated sample (light bars) compared to the non-BuDNJ control (heavy bars). A range of dilutions was done for each experiment but only one data point is shown here. Binding of ADP358 was similarly depressed at all dilutions tested.

mational changes in the molecule. We found the immunoreactivity of the fractions to a V3-loop-specific monoclonal antibody to be considerably different, but reactivities to a carboxy-terminal peptide-specific monoclonal antibody were unaltered (see figure).

These results suggest an alteration of the local conformation of gp120 in the region of the hypervariable V3 loop as a consequence of BuDNJ-induced hyperglycosylation. On the basis of homology with protease inhibitors⁸, it has been suggested that the V3 loop is cleaved during viral entry and that this could be a necessary part of the infection process⁹. It is tempting to speculate that the altered V3 loop conformation induced by treatment with BuDNJ cannot be cleaved by the normal cellular tryptase-like enzyme, and that fusion of the viral and cellular membranes is thus prevented. Lack of syncytium formation is the observed phenotype of BuDNJ-treated HIV cultures. Understanding the subtleties of the HIV infection process may yet contribute to the design of other clinically useful anti-viral agents.

IAN M. JONES

NERC Institute of Virology,
Mansfield Road,
Oxford OX1 3SR, UK

GARY S. JACOB

Monsanto Company,
800 North Lindbergh Blvd,
St Louis, USA

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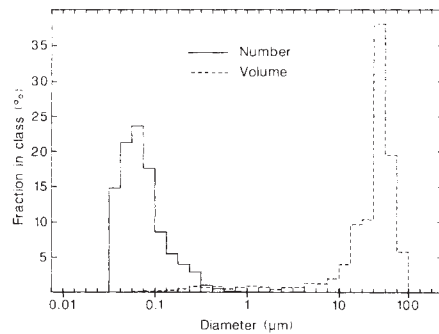
Risks to VDT operators

SIR — Ziembra in Scientific Correspondence¹ reports high α -activities on the surface of video-display-terminal (VDT) screens. He attributes these activities to radon progeny deposition due to high levels of electrostatic charge on the screen, and suggests that such deposits are the cause of some common health complaints from VDT operators. My studies of particle deposition in offices with VDTs²⁻⁴ have demonstrated that in the presence of static electricity, aerosol particles will accumulate at increased rates both on the VDT screen and, more important, on the face of the VDT operator, providing a more likely explanation for some health problems. I believe that in the absence of an electrostatic field the human face will be partially protected from particle exposure because of thermophoresis — a repulsive action of the temperature gradient at the surface of the skin.

The cathode ray tube of many VDTs carries a net charge due to the high electrostatic voltage applied on the inside. My survey³ of Norwegian offices, for example, revealed that more than a third of the VDTs in use exhibited effective surface potentials in excess of 3,000 volts. The electrostatic conditions of such environments are often aggravated by triboelectric charging of the human body, which in extreme cases may lead to positive or negative body potentials of several thousand volts. Such phenomena can give rise to electrostatic fields of the order of 10^2 V cm⁻¹ near the VDT as well as the face³. Airborne particles carrying a charge, as most do, will be induced to move if subjected to fields of such intensity. The drift velocities of these particles are typically in the range 0.01 to 0.1 cm s⁻¹ and thus much smaller than normal indoor air velocities of some tens of cm s⁻¹. Nevertheless, particles with the appropriate charge polarity and passing sufficiently close to a charged surface are likely to be captured.

I have analysed such particle deposits by scanning electron microscopy and have found that, in terms of numbers of particles, the deposits consist dominantly of sub-micron particles (see figure). Deposition rates well in excess of 10^5 particles cm⁻² h⁻¹ have been registered both on solid objects and on the face of a human subject⁴. In these experiments, the rate of deposition on charged surfaces exceeded that on nearby neutral surfaces by a factor of at least five. The figure also shows that the particulate volume (and thus mass) of electrostatic deposits is primarily made up of coarse particles. A rough calculation based on the data represented by the figure suggests a mass deposition rate of about $0.05 \mu\text{g cm}^{-2} \text{h}^{-1}$, of which the submicron fraction comprises only about 4 per cent.

The range of α -radiation from radon progeny is small compared with the VDT/



Size distribution of particles accumulated on vertically oriented, charged surfaces in office environments. Data from several deposition experiments have been combined to generate the size spectrum shown. The deposits were collected on thin polycarbonate substrates and analysed using a scanning electron microscope. Particle size and volume are defined as the diameter and volume, respectively, of a sphere of equivalent projected area. The set of magnifications used corresponds to a particle detection limit of 0.03 μm . Solid line, number of particles; broken line, volume of particles.

operator separation, which usually exceeds 30 cm. It is not easy to see, therefore, in what way a particle deposit on the VDT can be of harm to the operator. Problems may be anticipated, however, if particles accumulate directly on the operator's face. In 1981 I suggested² that such particle exposure is the cause of a certain type of facial rash experienced by VDT operators⁵. The detailed aetiology of these rashes has

not yet been resolved.

Published research on the subject of facial particle exposure is scarce. I imagine that the temperature of the human body will be an important factor. It is known that heated solid objects are less prone to contamination from airborne particles due to thermophoresis (a repulsive force arising from the temperature gradient at the surface). As this effect will be significant at temperature differences comparable to that of the human skin/ambient air combination⁶, it is conceivable that the human body is similarly protected. It is known, however, that the effects of thermophoresis are readily overcome by electrostatic forces of even moderate strengths⁷. Environmental static electricity may therefore influence human health to a greater extent than has hitherto been recognized.

WALTER C. WEDBERG

Engineering Centre,
Department of Physics,
University of Bergen,
Bergen, Norway

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Stimulation by superantigen

SIR — Kawabe and Ochi show¹ that mature CD4⁺ peripheral T cells bearing the V β 8 antigen receptor are initially increased and then decreased after injection of the superantigen SEB into mice. The authors believe that this represents a deletional mechanism in these cells, due to receptor-mediated induction of programmed cell death (apoptosis) by interaction with the superantigen. They infer this from the observation of DNA fragmentation in V β 8⁺ T cells. We suggest a different interpretation.

Some of the DNA in T cells taken from animals four days after SEB injection was fragmented into nucleosomes. At four days the peak of the increase in V β 8⁺ numbers occurs, and it is odd that DNA fragmentation is seen then, and not at day two or day seven. If abnormal stimulation of T cells caused their death one might expect them to die early, within 24 hours (ref. 2). We suggest that V β 8⁺ cells were stimulated by the superantigen SEB to proliferate between days zero and four. Around day four the SEB concentration would have declined below the level required for continued stimulation. T cells that had become dependent upon T cell lymphokines (autocrine or paracrine) would stop proliferating and then die

by apoptosis. We have shown³ that the removal of growth factors such as interleukin-2 will cause apoptosis in dependent cells.

The process of DNA fragmentation takes about 12 hours to become obvious after lymphokine depletion, and it precedes cell lysis by several hours. Apoptotic cells and their nuclei are fragile⁴. It is likely that the fairly vigorous cell preparation procedures used by Kawabe and Ochi removed most of the apoptotic cells so that no fragmented DNA was observed at time 0. However, incubation *in vitro* in the absence of lymphokines would have allowed apoptosis to proceed to a level detectable by electrophoresis, a method that is sensitive although not quantitative (see Fig. 2 of ref. 1).

The peripheral deletion of antigen-responsive cells may be possible. It must be a most interesting process by which a mature T cell, interacting with antigen in the periphery, decides whether to respond and, if not, whether to linger or die. But we think Kawabe and Ochi present insufficient evidence to postulate direct antigen receptor-mediated induction of apoptosis in mature

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V β 8⁺ T cells by SEB or SEB-MHC class II complexes.

J. JOHN COHEN
RICHARD C. DUKE
KAREN S. SELLINS

Department of Microbiology
and Immunology,
University of Colorado Health Sciences
Center,
Campus Box B-175,
Denver,
Colorado 80262, USA

Spontaneous mutation rate

SIR — We have described a mouse *in vivo* mutagenicity assay capable of detecting mutations at the *Dlb-1* locus which encodes the *Dolichos biflorus* lectin receptor expressed in the epithelium of the small intestine¹. In this intestinal mutagenicity test (IMT), clonal descendants of embryonal intestinal stem cell precursor cells mutated *in utero* can be identified histochemically as unstained groups of enterocytes on villi in otherwise positively stained epithelium of adult animals (8–12 weeks of age) using peroxidase-labelled lectin. The test permitted us to quantify spontaneous mutation rates in untreated mice as well as rates of induced mutations. Our results, derived from scorings of intact preparations (Fig. 1) of complete small intestines, indicated that spontaneous mutation rates are considerably

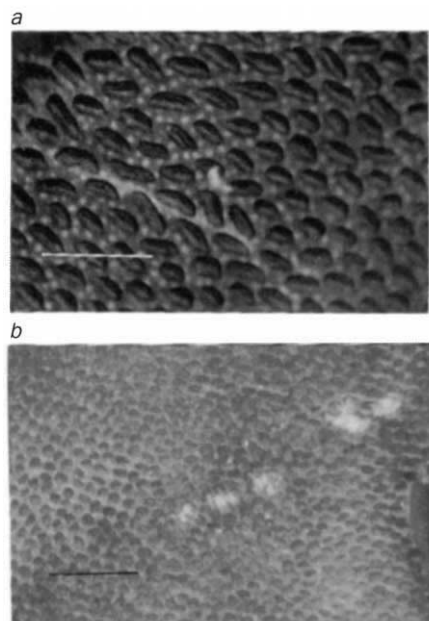


FIG. 1. Regions of duodenum from ENU-treated *dlb-1^a/dlb-1^b* mouse *in utero*. Most of the villi appear brown following histochemical staining using peroxidase-labelled *Dolichos biflorus* agglutinin. Fully separated and mucus-free gut preparation showing a mutated crypt (arrow) bearing the *dlb-1^a/dlb-1^b* genotype feeding two distinct adjacent villi. Mutated villi occurring in distinct patches of villi which fail to bind the lectin, thus appearing unstained. Scale bars: a, 0.5 mm; b, 1 mm.

lower than reported by Winton *et al.*², who used mice aged 3–26 weeks in a similar biological test system involving different methodological approaches. We therefore reassessed levels of spontaneous mutations at the *Dlb-1* locus by scoring whole-mount preparations of the mouse gut as described¹, using mice aged up to 56 weeks (Fig. 2). The slope of the curve was estimated to be 0.01, whereas a slope of 0.27 was estimated from Fig. 2 of Winton *et al.*². We estimate a spontaneous mutation rate of 0.5 mutations per 10⁴ villi per year, whereas the estimate given by Winton *et al.* is 13.5 mutations per 10⁴ villi per year.

Although the reasons for this discrepancy are unclear, it seems that the different methods used to carry out the assay may have contributed to the considerable differences in the results obtained. The large standard deviations reported by Winton *et al.*² (their Fig. 2) may reflect technical difficulties in scoring subsamples of gut preparations (about 5 per cent of the total gut) to determine spontaneous mutation rates. By contrast, we have shown that because of the sporadic distribution of mutant villi and mutant villus patches in the gut it is essential to score the entire intestine and to generate undamaged gut preparations in which the histochemical staining extends from the crypt orifices to the tips of the villi¹ (Fig. 1). We have therefore developed methods that eliminate sample variability associated with subsampling, incomplete tissue staining and sporadic tissue damage. Our method is reliable, as shown by the minuscule standard deviations of our data (Fig. 2).

Mutant villi occur either isolated or in small and spatially isolated patches¹. The former arise as a result of mutations in stem cells in individual crypts² or in embryonal intestinal precursor cells. Their descendants are thought to be deposited in neighbouring or closely adjacent crypts, thus causing the appearance of patches of mutant villi. Thus, in assessing the number of mutational events in gut preparations we have scored mutant villus patches as single mutational events rather than counting each mutated villus within a patch separately¹. Winton *et al.* scored unstained ribbons on immediately adjacent villi arising from an apparent common centre (a crypt) resulting from single events, whereas villi in patches that were not immediately adjacent were scored as resulting from various events. Adult guts display both mutated villus patches as well as single mutated crypts stemming from single mutational events occurring during embryogenesis or in adult mice, respectively. Therefore, the scoring technique used by Winton *et al.* presumably contributed to an inflated estimate of spontaneous mutation rates.

This suggestion is strengthened by a recent publication by Winton *et al.*³, who scored numbers of mutated crypts from the abluminal side of the gut, rather than mutated ribbons on villi, which led to estimates of the spontaneous mutation rate of about 10-fold

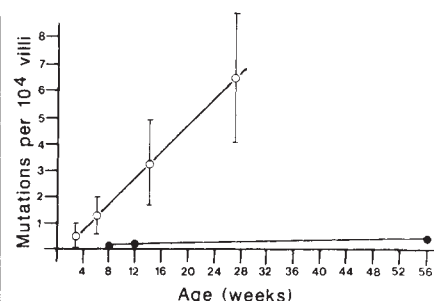


FIG. 2 Whole-mount preparations of small intestine (pylorus to ileum) from four mice were scored each at 8 and 12 weeks of age and three intestines at 56 weeks. Mutations were observed as spatially isolated patches of one to two striped villi which were scored as separate mutation events. The total number of villi was estimated by taking measurements from four intestinal preparations. Using this estimate, the mutation scores per intestine were adjusted to mutations per 10⁴ villi and plotted against time. Data are presented with error bars smaller than the symbols used (●). The resulting slope fitted to our data is 0.01, whereas that of Winton *et al.*² (their Fig. 2) is 0.27 (○).

lower than in their earlier studies² (assuming an average of 10 crypts per villus⁴), and thus are similar to our observations¹. Additional inconsistencies exist between results of Winton *et al.*^{2,3} regarding mutation rates induced in response to ethylnitrosourea (ENU) (50 mg kg⁻¹) treatment, differing about 50-fold depending on their scoring procedure. Such discrepancies emphasize the need for a standardized scoring procedure to determine quantitatively the potential of mutagenic agents in this assay, particularly if used for human risk assessments.

Using a different approach, Griffiths *et al.*⁵ observed no spontaneous mutations in the colon using glucose-6-phosphate dehydrogenase as a marker gene in sections containing about 400,000 crypts. Even though these results may not be comparable directly with our data, they are not surprising: assuming an average of 10 crypts per villus⁴, our estimate of 0.5 mutations per 10⁴ villi per year may be converted to 0.5 mutations per 10⁵ crypts per year. Hence, there is little chance of observing significant numbers of mutated crypts in a sample of 400,000 and even less so considering that the age of the mice used by Griffiths *et al.* was less than one year⁵. Our findings corroborate those of Griffiths *et al.*, indicating that the rate of spontaneous mutation in the intestine is extremely low.

JOHN F. O'SULLIVAN
GÜNTHER H. SCHMIDT*
DIETER PAUL

Department of Cell Biology,
Fraunhofer Institute for Toxicology
and Molecular Biology,
Nicolai-Fuchs-Strasse 1,
3000 Hannover 61, Germany

*Present address: Merck and Co. Inc., Growth Biochemistry and Physiology, PO Box 2000, Rahway, New Jersey, 07065, USA.

WINTON *ET AL.* REPLY—O'Sullivan *et al.* challenge our estimate² of the rate of spontaneous mutation at the *Dlb-1* locus in intestinal epithelium. We suspect that the apparent discrepancy between their results and our own may be due to the scoring of two different types of mutant clone. O'Sullivan *et al.* score the large descendant clones induced by mutation during embryonic development, whereas we are scoring the smaller clones arising from mutation of individual crypt stem cells in adult mice. Other of their criticisms seem to arise from misunderstanding. Our view has consistently been that the best way to resolve the disagreement would be for us to compare experimental material. Unfortunately, O'Sullivan *et al.* have not agreed to this course of action.

Taking their criticisms in turn: (1) Our finding of more mutations is said to be due to poor methodology: tissue damage, incomplete staining and sampling error. We refute this. In all our experiments, heterozygous mice and control B6/B6 (*Dlb-1^b/Dlb-1^b*) homozygous mice are scored blind. We have never found mutations in the homozygous controls, which argues strongly against our having scored artefacts as mutations. In addition, we obtain a linear accumulation of mutations with age in untreated mice, and consistent dose-response curves for several different mutagens: unlikely if we were scoring artefacts. We have preferred to sample a large number (12 mice) at each time point. If we were scoring descendant clones like O'Sullivan *et al.*¹, we would need to score the entire intestine because these clones are so much less frequent (three or fewer per mouse in their material). In fact, the physiological and anatomical differences between different regions of the small intestine suggest that, in mutation experiments, these regions may be best treated separately⁶.

The standard deviations of our measured rates are due partly to Poisson variation associated with counting, and partly due to variation between animals. The contribution of Poisson variation to the rates at later ages is quite small, since these are based upon relatively large counts. Of course, at younger ages, where mutations are less frequent, Poisson variation is a greater source of variability and there would be something to be said for sampling a larger part of the gut. However, O'Sullivan *et al.* are wrong in suggesting that such variation is excluded by counting the whole gut. The precision of their method, like ours, is limited by the number of counted events upon which the estimates are based. They give us no information about this. Their 'minuscule' standard deviations may or may not be impressive; it must be remembered that these apply to much smaller rates. A fairer comparison would be provided by the coefficient of variation.

(2) O'Sullivan *et al.* suggest that we are scoring the components of descendant clones as separate adult mutational events. This may occasionally be true; but most

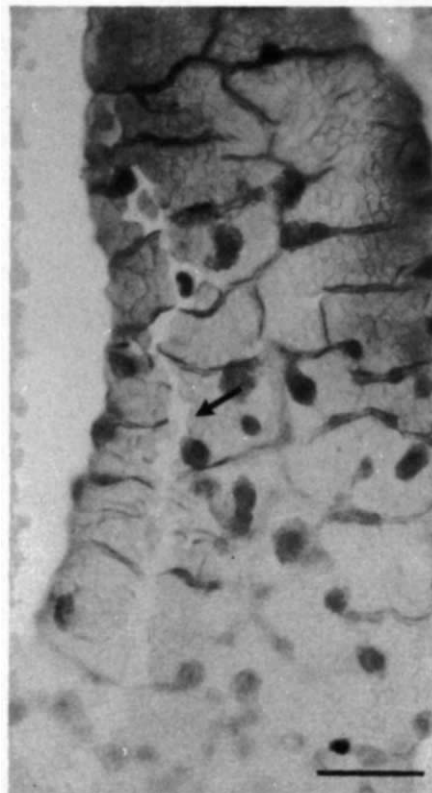


FIG. 3 A jejunal villus dissected from a small intestinal whole mount made from an adult B6 × SWR F1 mouse treated two weeks previously with ENU (50 mg kg⁻¹). The whole mount was stained with a peroxidase conjugate of *Dolichos biflorus*. Note the unstained ribbon of epithelial cells (arrow) which arises by mutation of a single stem cell and which is between one and two cells wide. Scale bar, 100 µm.

descendant clones are easily recognized by the clustering of mutant villi in a small area. O'Sullivan *et al.*'s own data (Table 1 in ref. 1) show that these clones are in any case very infrequent, and therefore will seldom fall within our sampling area. More important, counting of descendant clones cannot explain our observation of the linear increase in mutations with age, which is the main point at issue.

(3) O'Sullivan *et al.* suggest that our published results show large inconsistencies. The comment that our ethylnitrosourea (ENU) experiments show discrepancies of 50-fold in mutation rates shows a misunderstanding of the data. The cells in the base of the crypt turn over slowly, and it takes 12 weeks to see mutated crypts³ as opposed to the two weeks to see mutated villus ribbons. The data are therefore not inconsistent, but as one might expect from the generally accepted model of crypt organization. There is one, much smaller, real difference between the mutation rates scored on villi and in crypts. There are two *Dlb-1* mutant phenotypes: one which results in complete loss of DBA-peroxidase binding (and thus of staining) in crypt and villus cells, and one which is expressed as loss of staining on the villi only. These occur in different ratios following treatment with dif-

ferent mutagens in B6 × SWR F1 mice. When these phenotypes are allowed for, there is no discrepancy between mutations scored in crypts or on villi.

The comparison with the results of Griffiths *et al.* may not be relevant because they were scoring a different epithelium in different mice with a different marker, mutation of which might well confer a selective disadvantage. Other studies in human colonic epithelium, using a mucin marker more comparable to *Dlb*, have suggested a significant accumulation of mutations⁷.

In conclusion, O'Sullivan *et al.* would like to develop the *Dlb-1* assay to detect mutation of embryonic progenitor cells and the resulting descendant clones. But they have not shown that they can detect the much smaller clones which arise by mutation of adult renewing stem cells in fully developed small intestine (Fig. 3). In respect of the latter we have demonstrated dose responses in the induction of *Dlb-1* clones for ENU², irradiation⁸ and several other mutagens including ENNG, MNNG and MNU (D.J.W. and B.P., unpublished observations). Until O'Sullivan *et al.* demonstrate that they can detect mutations occurring in adult small intestine, we suggest that the discrepancy in the estimate of the spontaneous mutation rate in adult mice between our two groups may result from the low resolution of their scoring method.

We find it surprising that O'Sullivan *et al.* challenge our data when they present so few of their own. Their published data are confined to the description of a few descendant clones, and the data they present here in relation to mutations in mice over 12 weeks of age come from three mice at one time-point in one experiment. Even so, we take their comments seriously and are anxious to know if there is really a discrepancy due to some factor or factors which we have not identified. Unfortunately, the inadequate description of their scoring method and their reluctance to compare experimental material make it likely that the problem, if there is one, will remain unresolved.

DOUGLAS J. WINTON

M. A. BLOUNT

BRUCE PONDER

CRC Human Cancer Genetics Research Group,

Department of Pathology,
University of Cambridge,
Cambridge CB2 1QP, UK

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Olbers' paradox in concert halls?

SIR — There are some ways of looking at problems in physics that transcend the fields in which they originated. An important case is statistical mechanics, developed in the latter half of the nineteenth century for the kinetic theory of gases.

But traps await those who do not comprehend the limitations of the original theory. The kinetic theory cannot apply to systems the size of the mean-free-path between collisions of molecules, 10^{-7} m at atmospheric pressure, or 10^{-1} m in a good vacuum, for collisions with the boundaries then predominate.

In about 1900, W. C. Sabine attempted a similar theory of room acoustics, calculating a 'mean-free-path' of fronts of sound between their encounters with the boundaries of rooms. This path was of the order of the size of the room; its exact length was the subject of controversy for 75 years, for the mathematics are formidable. There is no unique and singular path length, as W. B. Joyce showed in 1975¹.

Recently, a stronger reason for abandoning Sabine's model has been found. The presence of even moderate amounts of sound-absorbing materials in a room makes impossible the uniform field of energy, slowly varying with time, that Sabine supposed². The audience in a concert hall (or any auditorium) is highly absorbing, so the existence of such a field is out of the question. The physics of concert halls is the physics of flows of energy and information from the orchestra to the audience. The correct theory is based on physical acoustics and the encoding of information in transient structures of waves: the physics pioneered by Denis Gabor³.

What has this to do with Olbers' paradox, the question of why the sky is dark at night? If the stars were eternal and the Universe infinite, space would be filled with a uniform luminosity, for every line of sight would intercept the surface of a star. This problem has engaged astronomers for 400 years. The solution accepted now is that the time required for a uniform field to develop is much longer than the lifetime of galaxies. It is not necessary to invoke relativity or an expanding Universe⁴.

The existence of strong absorption in

the Universe is not so clear as in concert halls. Its presence makes the 'time-constant' so short that too few reflections occur to generate a uniform field; the number of these reflections is inversely proportional to the strength of the absorption. Surprisingly, neither scale nor speed of propagation makes a difference; the same relation obtains for molecules in a microvacuum, sound in a concert hall or light in the Universe.

In this sense, a time-varying classical Universe is like a time-varying classical concert hall. The mediaeval idea that music and astronomy are closely related is true in this case. Although Sabine's theory clearly is wrong, he instituted another paradox by using it to design the world's finest concert hall, Boston Symphony Hall. I speculate that he drew on the earlier, correct approach to the problem by Joseph Henry⁵.

For nearly 100 years we have suffered concert halls designed to a wrong theory. No designer since Sabine has mastered the art, so the thousands of auditoria built each year are, at best, mediocre. A good theory will have a great practical effect.

JAMES B. LEE

Department of Mechanical Engineering,
Portland State University,
Portland, Oregon 97207-0751, USA

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Object awareness

SIR — Libet¹ makes three comments about our work. (1) We do not dispute that some forced-choice perceptual tests can be performed successfully without awareness^{2,3}, but this is irrelevant to our recent claim⁴. We described tests of visual perception of orientation, shape and size which our brain-damaged patient (D.F.) failed; she failed them whether we asked her for verbal reports, or for manual pantomime judgements, or for forced-choice judgements. In short, on all the perceptual tests we used, there was no evidence for awareness of these object qualities. By contrast, when we asked her to act on the objects, she did so with great success and with apparently normal skill. It was this remarkable dissociation between D.F.'s perceptual judgements and her visuomotor control that led us to postulate a separation in the underlying neural architecture.

(2) Libet then argues that such a separation of brain systems for perception and action need not imply a general

separation between conscious and unconscious functions. Again this is uncontroversial. We were making the much more specific suggestion that D.F.'s profound visual-form agnosia⁵ might be caused by the destruction or disconnection of perceptual systems that normally mediate conscious visual-object perception; and that her preserved visual skills are probably exploiting relatively intact structures which in the normal individual mediate the visuomotor control of prehension and other motor acts.

Of course, unlike our patient, normal subjects can have conscious 'perceptual' information about the visual objects and targets which they grasp or foveate; but we believe that the processing mediating that experience is distinct from the visuomotor computations that modulate the movements themselves. In fact, just like D.F., normal subjects may generate visually guided eye and limb movements on the basis of covert visual information that is inaccessible to consciousness⁶. Indeed, we suspect that an important feature of conscious visual perception is that it can remain refractory to the visual information to which visuomotor networks are often explicitly sensitive.

(3) Finally, we do not deny that in some cases information processing might become conscious or unconscious simply as a function of the duration of relevant afferent neural activity. But such a conclusion is by no means forced on us by Libet's experimental data. The fact that a single thalamic pathway was electrically stimulated in his two experimental conditions (long versus short duration) does not ensure that one and the same perceptual system was activated in the two conditions of stimulation. Furthermore, the distinction between brief and longer-lasting activation cannot account for the dissociations we observed in D.F.; there is no plausible reason to suppose that visual pathways were activated more briefly in one type of task than in the other. Indeed, the objects whose qualities she failed to perceive, but was able to act on, were always present for at least several seconds, and sometimes for up to a minute.

M. A. GOODALE
A. D. MILNER
L. S. JAKOBSON
D. P. CAREY

Psychological Laboratory,
University of St Andrews,
St Andrews, Fife KY16 9JU, UK

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Correction

The first sentence of the letter from N. W. Pirie (*Nature* **351**, 704; 1991) should read: "By using the title 'Sperm warfare', Daedalus' research team emphasizes that it thinks only of aggression as a factor in sperm competition." The incorrect Daedalus title was an editorial error. □

A little man made good

Roy Porter

Edward Jenner 1749 – 1823. By Richard B. Fisher. *André Deutsch*. Pp.341. £17.99.

EDWARD Jenner: biographer's dream or nightmare? He is, in some ways, the most commemorable figure in the entire history of medicine, for smallpox vaccination has surely saved more lives than any other prophylactic breakthrough, and the global eradication of the disease within recent times is a success story without equal. And he remains a heart-warming prototype of the 'little man made good'. Jenner, after all, was no eighteenth-century precursor of a grandee, grant-swinging professor or research-director; he was, rather, a retiring, slow-talking country doctor, practising in darkest Gloucestershire, whose earlier enquiries had been into the hibernation of the hedgehog and the murderous habits of the cuckoo. His great discovery arose, endearingly and edifyingly, from attentiveness to humble things; milkmaids who had contracted cowpox from infected udders subsequently escaped, he noticed, the ravages of "the speckled monster". This was widespread country wisdom, but Jenner had the curiosity to turn it to science, grasping the immunizing properties of the bovine disorder, and displaying a fine practical bent for developing a safe and simple variolating technique.

Yet Jenner also poses his biographer intractable problems. For one thing, he was not a man who thought with his pen (he often employed literary assistants). Though he lived more than 70 years and achieved immortality, long passages of his life remain ill recorded. He spent the bulk of his days, before and after the wonder years around 1800, engaged in the routine daily calls of a provincial practitioner, tending sore throats and sick babies. Precious little is known about his inner musings, or his relations with his deeply pious wife and his children, beyond the general impression of a conventional gentleman, temperamentally well suited to village obscurity down by the Severn Estuary.

And how can one avoid the feeling of anticlimax? John Hunter, Louis Pasteur, Robert Koch – all had careers of lasting creativity, studded with multiple discoveries; Jenner, by contrast, was a one-hit man. He was, as Richard Fisher, his latest biographer, ruefully notes, an ordinary man struck by one colossal lightning-bolt of serendipity.

Fisher has trawled some new materials, but they have not led to any dramatic revelations. Hence, it is not surprising that his telling, though agreeably written, does not wholly overcome the problem of sustaining interest over long stretches of rather humdrum doings. But he does offer a convincing reading of Jenner's rather ambivalent

IMAGE
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REASONS

Jenner — an ordinary doctor struck by one colossal bolt of serendipity.

character. What should have been Jenner's decade of triumph turned bitter upon him. He was soon being fêted in London. Parliament voted him an award of £10,000 – a cool million or two in modern values – and when the Royal Jennerian Society was founded in 1802, he was made president of its medical council. (What other doctor had a royal society named in his honour during his lifetime?) Nevertheless, the letters of this period are those of a soul in purgatory. Why?

Catapulted to greatness, Jenner found it hard to handle. Prompted equally by duty and pride, he wished to be at the thick of things in London, promoting vaccination, or at least stopping such rivals as George Pearson from stealing his thunder. But he also longed to slip

back into "domestic peace" and rustic anonymity. He wasn't psychologically smart enough to elevate himself, like a figurehead, above controversy; but his interventions in the business of the metropolitan vaccination institutions proved damagingly disruptive, and he had no head for bustle or bureaucracy. He was a man torn and troubled.

Above all, he could not cope with criticism. Glorifying in the role of benefactor of mankind, Jenner was inclined to treat all doubts about vaccination as personal insults to his probity, the splenetic outpourings of cranks and mercenary inoculators. Partly for this reason, he was never able to face head-on the big question: did vaccination, like inoculation, confer life-long immunity? Thus pressurized, he grew more tetchy, even (suggests Fisher, maybe using too strong a term) "paranoid".

Why such acute sensitivity? Fisher comes up with two plausible suggestions. Jenner was a truly tender man. Every reported smallpox death of a vaccinated subject hurt him to the quick. But he also had his fair share of vanity. Perhaps spoilt by his parents, he had certainly become, while studying in London, the great John Hunter's darling pupil, and never lost the backing of that founder of scientific surgery. Though rather indolent by disposition, he nevertheless expected to step into his master's shoes, and so viewed the world's plaudits as no less than his due. Unlike Hunter himself, he had no salutary schooling in adversity.

Aside from the light thus thrown on Jenner's psyche, this book tells us much about the milieu of medical science around 1800. The Georgian century is sometimes dismissed as a fallow period in medical inquiry, in which Jenner shines out as some lone genius. But the truth, as Fisher rightly perceives, was far different. There

was a healthy buzz of medical inquiry around Jenner's Gloucestershire – Thomas Beddoes (of TB fame) in Bristol, Caleb Parry in Bath. Such provincials achieved an *esprit de corps* through medical societies, and were in close touch with the capital, thanks, not least, to that new invention, the medical journal. They made the most of local opportunities to study epidemics and animal diseases. If Fisher cannot, alas, add much to the story as told for instance in Paul Saunders' *Edward Jenner: The Cheltenham Years* (University Press of New England, London, 1982), he is, at least, highly successful in setting his hero in his medical landscape. □

Roy Porter is at the Wellcome Institute for the History of Science and Medicine, 183 Euston Road, London NW1 2BN, UK.

Replacing religion

Charles H. Bennett

The Hour of Our Delight: Cosmic Evolution, Order, and Complexity. By Hubert Reeves. Freeman: 1991. Pp.256. \$17.95, £14.95.

A GLANCE at the index (Nazis, Neutrin... Open Universe, Oppenheimer, Optimism, Order, Organization) hints at the scope of this book, the English version of *L'heure de s'enivrer: L'univers a-t-il un sens?* (Editions du Seuil, 1986). The superficial impression is of a cobbling together of two familiar but incompatible genres: a stinging indictment of civilization's destructiveness is followed by a careful elementary exposition of thermodynamics and cosmology. But the actual aim of the book is to fashion, out of the world view offered by modern science, a replacement for the sense of purpose formerly provided by religion and traditional myth.

By the early twentieth century, science had thoroughly undermined this sense of purpose by discovering the vastness of space, our random origin through chemical and biological evolution, and our apparent aloneness, but most seriously by predicting the eventual obliteration of all our accomplishments in a monotonous final state of thermal equilibrium. That gloomy prediction, Reeves points out, was based on the misconception of a static Universe: in the expanding Universe we now know we inhabit, entropy is diluted faster than it is produced, and we have, in principle, the possibility of an infinite event-filled future. Our thermodynamic death sentence commuted, things begin to look not so bad.

Indeed, Reeves makes them look rather traditional. 'Complexity', which the Universe produces in abundance as a side effect of its supercooling, takes over the job of good; our propensity and growing capability to destroy it take over from evil; and our fifteen-thousand-million-year gestation period and chemical kinship with all other matter and life give us a sense of place. Our divine mission? To assist the Universe in giving birth to further complexity, which we can do only if we outgrow our present tendency to self-extinction. How do we do this? Not by any hasty, zealous programme of action, but as a gentle side effect of contemplative delight in the Universe as we now understand it.

Except for the commutation of the thermodynamic death sentence, these ideas are not at all new; accordingly, Reeves' purpose in putting them in a book was not to propose them, but

rather to propagate them, especially to readers with no scientific background.

The book begins with the 'death urge', or tendency toward self-destruction, of our own or other technologically advanced civilizations, which may account for our not having heard any radio messages from them. This section is marred by a self-castigating anthropocentrism (for our senselessly cruel wars, environmental abuse and the unstoppable development of nuclear weapons) and romanticization of nature (where male seals fight, but do not kill one another) which in my opinion only distracts the reader from understanding the interplay of creative and destructive processes, both human and non-human, in the Universe as a whole. A less passionate discussion of, say, the genetics or economics of cooperation versus competition, or of the biosphere's response to major insults such as nuclear war or the events leading to mass extinctions in the past, would have been more helpful.

The next section, on the Universe's 'life urge', or complexity-generating tendency, is much better. While not attempting to define complexity itself, Reeves gives a thorough account of its creation in environments of gravitational, nuclear or chemical disequilibrium, as well as describing the supercooling which leads to these disequilibria. He codifies

key ideas in memorable symbols such as William Tell's arrow, which stands for a special initial condition inferred from its consequences.

With the help of this metaphor he treats the anthropic principle – the doctrine that physical laws or initial conditions of the Universe must be such as to have allowed the development of intelligent beings. Like most scientists, he regards it as a heuristic principle, rather than as a direct basis for inference, where it might serve as an unholy ally of the death urge, forcing us into existence against all odds, then abandoning us to our own devices.

Perhaps because I can no longer put myself in the place of a layperson, I found Reeves' explanations at times too simplified and didactic. I enjoyed the comparison of nuclear, atomic and molecular energy-level diagrams in the appendix, and wished he had found other ways to surprise and delight me with concrete examples of the manifold unexpected variety of the Universe while he was telling me about it. Unfortunately, this kind of pedagogic spectacle is much harder to produce in cosmological than in biological natural-history writing. □

Charles H. Bennett is at the IBM Research Division, T. J. Watson Research Center, Yorktown Heights, New York 10598, USA.

Chop and change

K. S. Bawa

The Conservation Atlas of Tropical Forests: Asia and the Pacific. Edited by N. M. Collins, J. A. Sayer and T. C. Whitmore. Macmillan: 1991. Pp.256. £65. Published in the United States by Simon and Schuster; \$95.

DESPITE worldwide concern, the rate of tropical deforestation is accelerating and in 1989 was almost twice that in 1979. (Norman Myers, *Deforestation Rates in Tropical Forests and their Climatic Implications*, Friends of the Earth, London). The pace of change is alarming, and only a small fraction of the original forests enjoy a conservation status. According to Collins, Sayer and Whitmore, the protected areas in seven Asian countries (Bangladesh, China (including Taiwan), India, Burma, Papua New Guinea, the Philippines and Vietnam) constitute less than 2.2 per cent of the moist tropical forest originally present. In two other countries, Indonesia and Malaysia, protected forests account for less than ten per cent of the original area under moist tropical forests. We know from species-area relationships that a 90 per cent reduction of the area roughly translates into the loss of 50 per cent of

the species. Thus, unless the protected areas can be increased and better secured, and degradation of the remaining areas prevented, thousands, perhaps millions, of species are doomed to extinction within a relatively short period.

Assault on forest ecosystems, as the editors of this book point out, stems from several sources: expanding human populations coupled with a shrinking natural resource base, misguided government policies, inappropriate models of economic growth, inadequate valuation of forest resources and logging without proper controls. Although several factors contribute to the current losses in biodiversity, there is little agreement about the extent of losses, relative importance of each factor and possible solutions to the problem. This volume represents an admirable effort on the part of the World Conservation Minorities Centre and the International Union of Conservation of Nature to present "objective and carefully researched information" to foster a constructive debate on conservation and management of forest resources. Collins *et al.* provide an overview of the extent of tropical forests in Asia and the Pacific, threats to these forests and prospects for their conservation.

The book is divided into two parts. The first part covers the general issues such as forest wildlife, shifting cultiva-

tion, management of natural rain forest, tropical timber trade, government policies, an action plan on tropical forestry, and the system of protected areas in the region. The concluding chapter of this section sums up the critical points: it is possible to enlarge the size of the protected areas, lessen the pressure on natural forests by raising plantations of fast-growing species, and manage natural forests on a sustained-yield basis. But these possibilities have always existed. Yet in recent years, the integrity of even the existing protected areas has not been maintained. Furthermore, there are few, if any, management plans for conservation of biological diversity in these areas.

Plantations of forest trees have consistently and significantly fallen below the established targets in many countries. Moreover, the plantations have failed to meet the needs of the rural poor, notably in India. Finally, although models of sustained-yield forestry exist, the assumptions of these models, as the editors themselves note, are often violated, leading to questionable management practices and forest degradation. Also, the claim that managed forests retain much of the original biodiversity is correct only under certain conditions. In summary, there are serious constraints on progress in conservation and use of forest resources, and a more extensive debate is needed on how such constraints might be overcome. Indeed, the stated aim of the book is to promote such a debate.

The second part provides information about problems and prospects of nature conservation in individual countries. Almost all major countries with significant amounts of tropical forests are covered. Their accounts provide an overview of the forests, biodiversity, existing and proposed protected areas, and conservation initiatives for individual countries. No other source offers such a comprehensive account for any of the countries of the region. Many chapters are written by, or based upon materials provided by the resident scientists of the countries concerned. Thus, the information is more credible than is usually the case in such general accounts.

Among the ten countries with the highest rates of tropical deforestation, five (Burma, India, Indonesia, Malaysia and Thailand) are in Asia. The book should provide a valuable and timely perspective to those engaged in finding a solution to the problems of deforestation and preservation of biodiversity. Conservation biologists should look forward to the publication of other volumes in this series □

K. S. Bawa is in the Department of Biology, University of Massachusetts, Boston, Massachusetts 02125, USA.

On shaky ground

David Swinbanks

Sixty Seconds That Will Change The World: The Coming Tokyo Earthquake.

By Peter Hadfield. *Sedgwick and Jackson: 1991. Pp.207. £17.60.*

WHEN the next major earthquake hits Tokyo, what will be the consequences for Japan and the rest of the world? This is the daunting question Peter Hadfield attempts to answer. He not only predicts major destruction and deaths within Tokyo but also portrays a picture of dire economic consequences for the whole world.



Earthquake drill — a shaking platform simulates the real thing for Tokyo inhabitants.

Much of what Hadfield says is based on the most recent evidence and reports available, as well as on extensive interviews with Japanese earthquake prediction and disaster prevention experts. Unfortunately the book, like its title, is dressed in sensationalism that may turn off the serious reader. This is a shame because he has a valid and very important message to put across.

Hadfield begins with a well documented description of the last major earthquake to hit Tokyo — the great Kanto earthquake of 1923, which killed about 140,000 people. He brings the full extent of that disaster to life with accounts of individual experiences based on newspaper reports from the time. And he brings home the terror of that day in 1923 when tens of thousands of people were roasted alive in Tokyo in fire storms that swept through the city after the earthquake.

He then argues that another major earthquake or string of earthquakes is

bound to hit Tokyo in the next few decades. Few would disagree with this prediction, which is based on the opinions of Japan's leading scientific experts in the field. But Hadfield then gets onto shaky ground by predicting the consequences of such an earthquake.

For this he relies rather heavily on a 1988 report by Japan's National Land Agency. This is the only government report to look in detail at the consequences for Tokyo and surrounding areas of a repeat of the great Kanto earthquake. The report predicts 80,000 to 150,000 deaths, depending on the time of day the earthquake strikes, and the destruction of millions of buildings by fire. Some experts say the report underestimates the full extent of the disaster;

others argue that most of Tokyo's modern buildings and roads, which have been built to withstand earthquakes, will fare better than predicted.

In the absence of a thorough independent assessment of the Land Agency report, its predictions are still open to question. But Hadfield then goes on to use a report produced by Tokai Bank, based on the Land Agency report, which says that a Tokyo earthquake will have disastrous economic repercussions for the world, and in particular the United States, because Japan will pull back hundreds of thousands of millions of dollars in overseas investments to reconstruct Tokyo.

Hadfield's gloomy picture may well be correct. But perhaps his greatest service has been to draw attention to a subject that requires much further study. □

David Swinbanks is Publisher and Tokyo Correspondent of Nature in Japan.

Recipe for bad science?

John Maynard Smith

Beyond Natural Selection. By Robert Wesson. MIT Press: 1991. Pp.376. \$29.95, £24.95.

AFTER *Beyond Neo-Darwinism* (M.-W. Ho and P. T. Saunders, Academic Press, 1984), we have *Beyond Natural Selection*. Wesson's book has the advantage over that edited by Ho and Saunders of the coherence that comes from being the product of a single author, although coherence is perhaps not the first word that springs to mind on reading it. Essentially, Wesson's argument is that the orthodox account of evolution in terms of natural selection of random genetic variation is inadequate, and must be supplemented by such concepts as self-organization, the autonomy of the genome, and the inherent tendency of organisms to evolve greater complexity. To support this, he adduces a series of familiar arguments – the inadequacy of reductionism, the gaps in the fossil record, complex adaptations that would be useless unless complete, selectively neutral variation and the persistence of apparently maladaptive characters.

How good a job does Wesson do? I will consider his treatment of sex and of social behaviour: both these topics have been the subject of much recent research by orthodox darwinists, essentially because both do seem at first sight to present challenges to their theory. If natural selection favours those types that multiply most successfully, why should two cells fuse to form one (sex) and why should some individuals not reproduce at all (sterile castes in social insects)? Does Wesson understand this recent research? Has he anything better in the way of explanation to offer?

In the case of sex, the answer to both questions is no. The chapter on sex contains much fascinating information. Unfortunately, there is no sign that he has read or understood any of the recent work on the subject. There is no reference to the books by G. C. Williams, Graham Bell or myself, and little mention of the ideas contained in them. There is no mention of the recent debate about the role of parasites in the evolution of sex and in sexual selection, or of A. Kondrashov's ideas about recombination and deleterious mutation. Wesson does not understand population genetics arguments. For example, he writes that "it is not known why inbreeding should necessarily be harmful". In fact, almost everyone agrees that a major cause of inbreeding depression is homozygosity

for deleterious recessives: the only debate is about whether this is the only cause. His confusion about inbreeding means that he does not understand the theories that have been proposed to explain the evolution of mechanisms, such as distyly in plants, that prevent selfing. At the end of the chapter, he writes (correctly), "Sex is not necessary to permit genetic change", but then spoils it by adding, "it seems somehow to make it more possible for organisms to change usefully". I take it that by 'organisms' he means populations; organisms do not evolve. But the real damage is done by the word 'somehow'; we understand very well why, and in what circumstances, sex enables a population to evolve more rapidly. Wesson ends by saying, "one must look for nondarwinian factors", but fails to come up with any suggestions. In fact, a number of plausible mechanisms for the origin and maintenance of sex have now been proposed; the problem now is to decide on the relative importance of these processes.

The chapter on social behaviour is more interesting. Wesson does not understand Hamilton's argument about inclusive fitness. He explains it quite well, but then continues, "It is not explained why sharing genes by immediate inheritance is more important than sharing genes by distant inheritance . . . The more homogeneous the population, the less significant is the familial relatedness of individuals."

I think of this, with affection, as 'Tinkle's fallacy'. Don Tinkle worked, at one time, on a parthenogenetic lizard consisting of a single clone of genetically identical individuals. He once asked me whether I did not find it puzzling that, if he put two females together in a cage, one would kill the other: after all, they shared almost all their genes – it is also the seventh of Richard Dawkins' "twelve misunderstandings of kin selection" (*Z. Tierpsychol.* 51, 184–200, 1979). But after this, the chapter improves. Wesson points out the difficulties that arise because of multiply mated queens, and nests with several queens, and argues for the importance of social dominance. Fair enough, but there is nothing remotely nondarwinian about social dominance. He then discusses the analogy between insect colonies and multicellular individuals, but misses the point that cooperation between the differentiated cells of an individual is stable only because all the cells arise from a single egg cell, and hence (apart from somatic mutation and occasional chromosomal elimination) are genetically identical. He does not offer any new ideas about the evolution of animal societies.

It is indeed the absence of new ideas that is so infuriating. Wesson appeals to

the 'autonomy of the genome' and to 'self-organization'. I do not know what these terms mean. The autonomy of the genome defeats me. At first sight it is manifest nonsense: no genome would get far without cytoplasm or an environment. Self-organization is equally trivially true. Of course an organism must organize itself: there is no-one out there to do the job for it, unless you imagine God painstakingly putting it together. Wesson also has a habit of explaining complexity by saying that things have an inherent tendency to become complex. This is precisely what Darwin objected to in Lamarck. The objection is that it explains nothing: it is like saying that a man is fat because he had a tendency to obesity.

Wesson uses one argument that deserves more attention than I have space for here. According to the anthropic principle, if (as we know to be the case) there are organisms able to think about their own origins, then whatever was necessary for their evolution must have been the case, however unlikely. There is a certain insane logic in this argument, but, if accepted, it spells the end of all attempts at historical explanation, including any attempt to explain evolution, which was a unique series of historical events. I will have to return to this topic some other time.

It is clear why Wesson wants to refute darwinism. He quotes Jacob Monod's remark, "Man at last knows that he is alone in the unfeeling immensity out of which he emerged by chance", and continues, "If we had, in the name of truth, to believe that humanity is the insignificant by-product of random change, selected by chance and material conditions, we should accept this valiantly and intelligently, although the truth could be dangerous for civilization and our well-being." (In passing, I think it is much more dangerous that a man should believe that he is uniquely favoured by God). Later, Wesson writes, "If one consistently adhered to the Darwinist canon, the logical social ethic . . . would be to join with genetically kindred persons to get the better, reproductively, of all others, ultimately to replace them by whatever means available." Because he does not want to be either despairing or selfish, he needs, if at all possible, to show that Darwin was wrong. This seems to be a recipe for doing bad science.

As Monod himself pointed out, we cannot derive our values from science: indeed, we cannot even do science unless we come to it with some prior values, not least a love of the truth. □

John Maynard Smith is in the Department of Biology, The University of Sussex, Falmer, Brighton, Sussex BN1 9QG, UK.

Interaction of *bride of sevenless* membrane-bound ligand and the *sevenless* tyrosine-kinase receptor

Helmut Krämer, Ross L. Cagan & S. Lawrence Zipursky

Department of Biological Chemistry, The School of Medicine and The Molecular Biology Institute, University of California, Los Angeles, California 90024-1737, USA

During development of the *Drosophila* retina, the R8 photoreceptor neuron induces a neighbouring cell to assume an R7 cell fate. Genetic data suggest that the induction is mediated by two transmembrane proteins encoded by *bride of sevenless* and *sevenless*. A direct interaction between these two proteins was demonstrated by the heterotypic aggregation of cell lines expressing them. In the developing eye the *sevenless*-dependent internalization of *bride of sevenless* by the R7 precursor cell provides evidence for a direct interaction between these two proteins *in vivo*.

INDUCTION of cell fate was first observed by Spemann and co-workers in amphibia more than half a century ago^{1,2} and is now known to be a broadly conserved developmental mechanism. Despite the importance of induction in development, we know little about the molecular nature of the inducing signals, the pathways that translate them into a cellular response, or the mechanisms by which the response to a signal is restricted to a subclass of cells. Recently, biochemical and genetic approaches have facilitated the identification of several inducers and their receptors. For instance, the fibroblast growth factor^{3,4} and transforming growth factor- β families^{5,6} are involved in the induction of mesoderm structures in amphibia; and ciliary neurotrophic factor induces the differentiation of type II astrocytes in the rat⁷. Although several putative receptors for inductive cues have been identified in *Drosophila*⁸ and *Caenorhabditis elegans*⁹, the ligands that activate them have yet to be identified.

Inductive interactions have been proposed to play a central part in the sequential determination of cell fate in the developing compound eye of *Drosophila*¹⁰⁻¹². The compound eye consists of about 800 ommatidia each containing a fixed number of cells, including eight photoreceptor neurons and several different types of non-neuronal cells. The eye primordium, the eye imaginal disk, is a columnar epithelium. Differentiation and pattern formation occur as a wave proceeding from the posterior to the anterior edge of the disk. The leading edge of the wave is referred to as the morphogenetic furrow. Anterior to the furrow, cells are undifferentiated and unpatterned, whereas immediately posterior to the furrow, cells assemble into clusters. Within these clusters the eight photoreceptor neurons differentiate stepwise in stereotyped fashion¹⁰. The step best understood is the induction of the photoreceptor cell R7.

Several genes have been identified that are directly involved in the establishment of R7 cell fate¹³⁻¹⁶. Mutations at the loci *sevenless* (*sev*), *bride of sevenless* (*boss*) and *seven in absentia* (*sina*) can prevent the differentiation of the R7 cell by causing a transformation of the R7 precursor into a non-neuronal cone cell (refs 16-18, and R.L.C., unpublished observation). The *sev* gene is thought to encode a receptor for an R7 inductive cue. The encoded protein (*sev*) is a transmembrane protein^{19,20} with

a cytoplasmic tyrosine-kinase domain²¹⁻²³. It is expressed in many cells in the developing epithelium^{19,20} but is required only in the R7 cell^{13,24}. The observed capping of *sev* in cell membranes next to the R8 cell led to the suggestion²⁰ that the ligand of the *sev*-encoded receptor is specifically expressed in the R8 cell.

The *boss* gene was thought to encode the ligand for *sev*, and hence the R7 inductive cue, on the basis of genetic mosaic analyses. These studies demonstrated a strict requirement for *boss* in the R8 cell¹⁴. Subsequent molecular analysis indicated that the *boss* gene encodes a protein (*boss*) with seven putative membrane-spanning regions and a large extracellular domain¹⁸. Here we show that *boss* is localized to the R8 cell, and we present immunohistological and *in vitro* evidence that *boss* is a ligand for the *sev* receptor.

Antibodies against *boss*

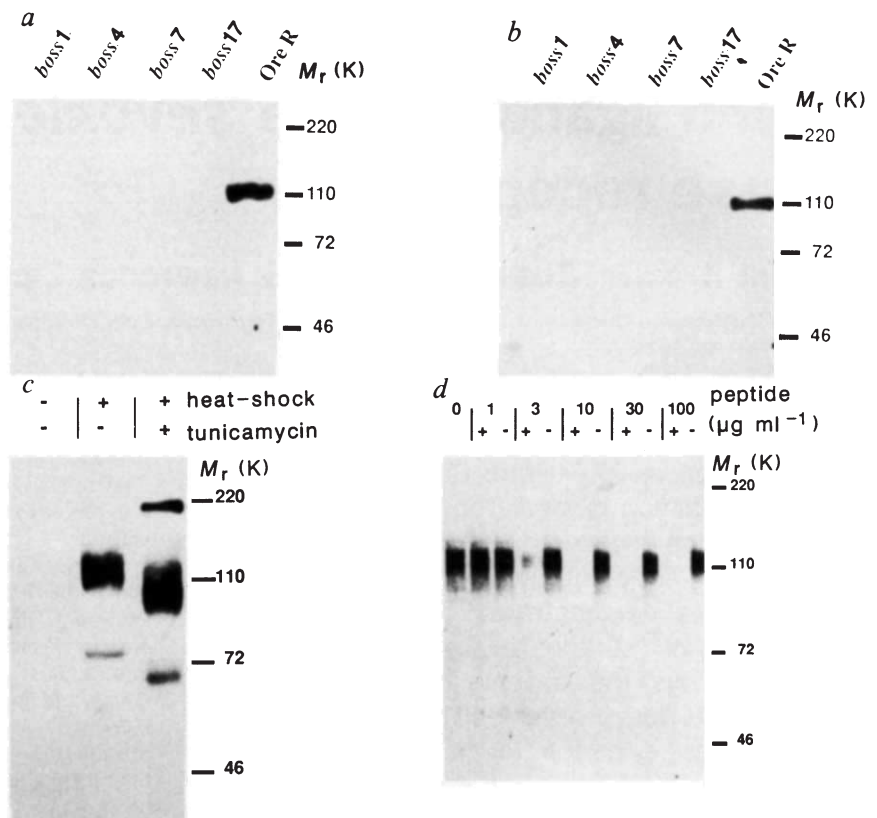
DNA sequence analysis indicates that the *boss* gene encodes a transmembrane protein with an extracellular domain of 498 amino acids, seven transmembrane segments and a cytoplasmic tail of 115 amino acids¹⁸. The *boss* protein is predicted to have a relative molecular mass of 96,000 (M_r 96K). Polyclonal and monoclonal antibodies against an N-terminal part of *boss* recognized a major band corresponding to an apparent M_r of about 120K on western blots of fly head extracts (Fig. 1a, b) and S2 cells expressing *boss* (Fig. 1c). The difference in M_r is most probably due to *N*-linked glycosylation, as *boss* expressed in S2 cells in the presence of tunicamycin, an inhibitor of *N*-linked glycosylation, had an apparent M_r of 100K (Fig. 1c). The specificity of the monoclonal antibody anti-*boss*1 was established by competition with the peptide *boss* 38-56 which consists of amino acids 38 to 56 of the *boss* protein (Fig. 1d). No immunoreactive product was detected in flies carrying one of four mutant *boss* alleles (Fig. 1a, b), which on the basis of their sequence are predicted to encode truncated proteins¹⁸.

Heterotypic aggregation of cells

To test whether *boss* and *sev* expressed on opposing cell surfaces bind directly to each other, we used a cell culture strategy successfully used by several groups²⁵⁻²⁸. When kept in suspension, S2 cells remain as single cells. Inducing *boss* expression in the stably transfected cell line S2: *boss*, in which a heat-shock promoter drives expression from a *boss* complementary DNA (Fig. 1c), did not increase the tendency of these cells to aggregate (Fig. 2a). Similarly, SL2-*sev* cells, which express *sev* constitutively under the control of the actin5C promoter²², remained as a single cell suspension (Fig. 2c; see also ref. 29). By contrast, when the SL2-*sev* and the heat-induced S2: *boss* cells were mixed together they aggregated to form clusters of variable size (Fig. 2b). Staining of these aggregates with anti-*boss* and anti-*sev* antibodies showed that the aggregates consisted of both cell types (Fig. 3). In control experiments, SL2-*sev* cells did not aggregate with heat-shocked S2 cells or uninduced S2: *boss* cells (data not shown). A second cell line expressing *sev* was obtained after transfection of S2 cells with a plasmid containing *sev* cDNA under control of a heat-shock promoter³⁰. Experiments with this

FIG. 1 Western-blot analysis of *boss* expression. The *boss* protein was assayed on western blots of homogenates from wild-type and mutant fly heads with affinity-purified rabbit anti-*boss* anti-serum NN-1 (1:2,000; *a*) or a hybridoma supernatant containing the monoclonal antibody anti-*boss*1 (α *boss* 1, *b*). Each lane contained the equivalent of one fly head (*c*) Lysates prepared from S2:*boss* cells were analysed on western blots with α *boss* 1. Each lane contained the equivalent of 10^5 cells. Heat-shocked treatments were for 30 min at 37 °C followed by a 2-h recovery at 25 °C. Where indicated, tunicamycin ($25 \mu\text{g ml}^{-1}$) was added to the cells 3 h before heat induction. The protein in the weak band corresponding to an M_r of 80K is not present on the surface of the S2:*boss* cells, because this band remained unchanged after trypsin treatment of intact cells. By contrast, the 120K band was sensitive to trypsin treatment (data not shown). *d*, To determine the amount of peptide *boss* 38–56 necessary to saturate the antibody binding sites (see quantification of aggregation in Fig. 2*d*), α *boss*1 ($40 \mu\text{g ml}^{-1}$) was pre-incubated for 30 min with the indicated amount of peptide *boss* 38–56 (+) or control peptide (–). These mixtures were used to detect *boss* protein expressed in S2:*boss* cells. M_r s are those of pre-stained standards (BRL).

METHODS. Plasmid pATH-*boss* NN encoding a *boss*-trpE fusion protein was obtained by cloning an 806-base-pair (bp) *NcoI*-*NheI* fragment of the *boss* cDNA, encoding amino acids 33 to 301 (ref. 18), into a slightly modified pATH3 vector. The resulting *boss*-trpE fusion protein was purified essentially as described²². This material was used into rabbits (0.5 μg) or mice (0.05 μg). Hybridoma supernatants were screened in a dot blot assay against the fusion protein⁵³. The N-terminal *boss* peptide *boss* 38–56 (TSPTKKSAPLRITKPQPTS); single-letter amino-acid code) and the unrelated control peptide (RPRITTRSPGFSPRVYN) were synthesized on a Applied Biosystems 430A peptide synthesizer with



T-boc chemistry by the UCLA peptide synthesis facility. Monoclonal antibodies or affinity-purified rabbit antisera were used for western-blot analysis⁵³ of homogenates from fly heads and S2:*boss* cells (see Fig. 2). Bound primary antibody was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; BioRad) and the ECL-Kit (Amersham).

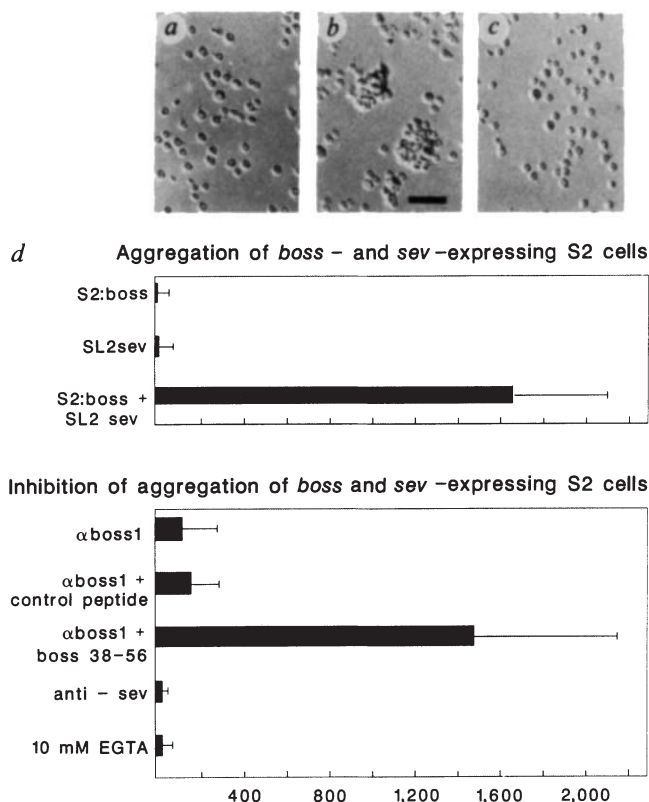


FIG. 2 Aggregation of S2:*boss* and SL2-sev cells. Heat-induced S2:*boss* cells (*a*) or SL2-sev cells (*c*) or a 1:1 mixture of the two cell lines (*b*) were incubated with constant rotation for 2 h at a density of 3×10^6 cells per ml. Aliquots were spotted on ceramic ring slides and photographed. Scale bar, 50 μm . *d*, Quantitative analysis of the aggregation of S2:*boss* and SL2-sev cells. Average numbers of aggregates exceeding a size of 50 μm from several experiments are indicated. Induced S2:*boss* cells, SL2-sev cells or a 1:1 mixture between the two cell lines were kept in suspension for 2 h ($n=5$). Where indicated, the aggregation of induced S2:*boss* and SL2-sev cells was performed in the presence of α *boss*1 ($40 \mu\text{g ml}^{-1}$) alone or with one of the two peptides ($30 \mu\text{g ml}^{-1}$; $n=4$), as indicated. The effects of goat anti-sev serum³¹ (1:200; $n=3$) or affinity-purified goat anti-sev antibodies³¹ (1:100; $n=2$) on aggregation were similar; the number given shows the average of the five experiments. An unrelated goat serum (1:200) showed no effect on the aggregation (data not shown). Where indicated, EGTA was added at 10 mM ($n=4$). Error bars indicate standard deviation. **METHODS.** SL2-sev cells²² and S2 cells were grown as described²⁶. A stable cell line expressing *boss* was established after cotransfection of 10^6 S2 cells with 10 μg pPC4 (ref. 54) and 20 μg pHT4-*boss* (D. Van Vactor *et al.*, manuscript in preparation) by the lipofectin method as recommended by the manufacturer (BRL) and limiting-dilution cloning²⁶. A cell line expressing a *sev* cDNA under control of a heat-shock promoter³⁰ was established, but this cell line was unstable and eventually lost. For the aggregation assay, cells were grown to near confluence. S2:*boss* cells were induced at 37 °C for 30 min. After 2 h at 25 °C cells adhering to the plastic flasks were washed and tritured into a single-cell suspension in Schneider's *Drosophila* medium (10% FCS) at a density of 3×10^6 cells per ml. After 2 h of constant rotation at 150 r.p.m., aggregates larger than 50 μm were quantified as described²⁷. Where indicated α *boss*1 ($40 \mu\text{g ml}^{-1}$) or α *boss*1 ($40 \mu\text{g ml}^{-1}$) after a 30-min preincubation with either the competing peptide *boss* 38–56 or the control peptide ($30 \mu\text{g ml}^{-1}$) or anti-sev antibodies³¹ were added to the cells immediately after they were mixed.

cell line also demonstrated binding of *boss*- and *sev*-expressing cells to each other (data not shown).

We quantified the aggregation of the *sev*- and *boss*-expressing cells by counting cell aggregates²⁷. Cell aggregates exceeding 50 μm (about five cell diameters) (Fig. 2a) were seldom observed in S2:boss or SL2-sev cells when incubated separately (Fig. 2d). When the two cell types were mixed, however, aggregates consistently formed (Fig. 2d). Addition of the anti-boss 1 monoclonal (40 μg ml antibody) to the aggregation mixture eliminated 90% of the aggregates formed (Fig. 2d). This inhibition was completely reversed by blocking the antibody with a 40-fold molar excess of the peptide boss 38–56 (Figs 2d and 1d). An unrelated peptide at the same concentration had no effect. Similarly, an antiserum raised against an N-terminal portion of *sev*³¹ inhibited the aggregation (Fig. 2d), whereas an unrelated goat serum used at the same concentration had no effect (data not shown). A requirement for Ca^{2+} in the binding of S2:boss and SL2-sev cells was suggested by the inhibition of binding by 10 mM EGTA, a chelator of calcium ions.

Internalization of boss by *sev*-expressing cells

Two characteristic asymmetries were observed in the interaction between the *boss*- and *sev*-expressing cells in culture. In many cases the *sev*-expressing cells extended their surface around the S2:boss cells, appearing to maximize the contact between the two cells (Fig. 3e, f). By contrast, the shape of the S2:boss cells appeared to be unaffected by the binding. A similar observation has been reported for *Delta*-expressing S2 cells when bound to *Notch*-expressing cells²⁸. In addition, in cell aggregates *sev*-expressing cells exhibited boss immunoreactivity; boss and *sev* colocalized to internal structures which we presume to be vesicles (Fig. 3). But *sev* immunoreactivity was not observed in the *boss*-expressing cells. This indicates that the SL2-sev cells can internalize boss. This internalization was *sev*-dependent, as boss-containing vesicles were not found in parental S2 cells when they were mixed or pelleted with S2:boss cells.

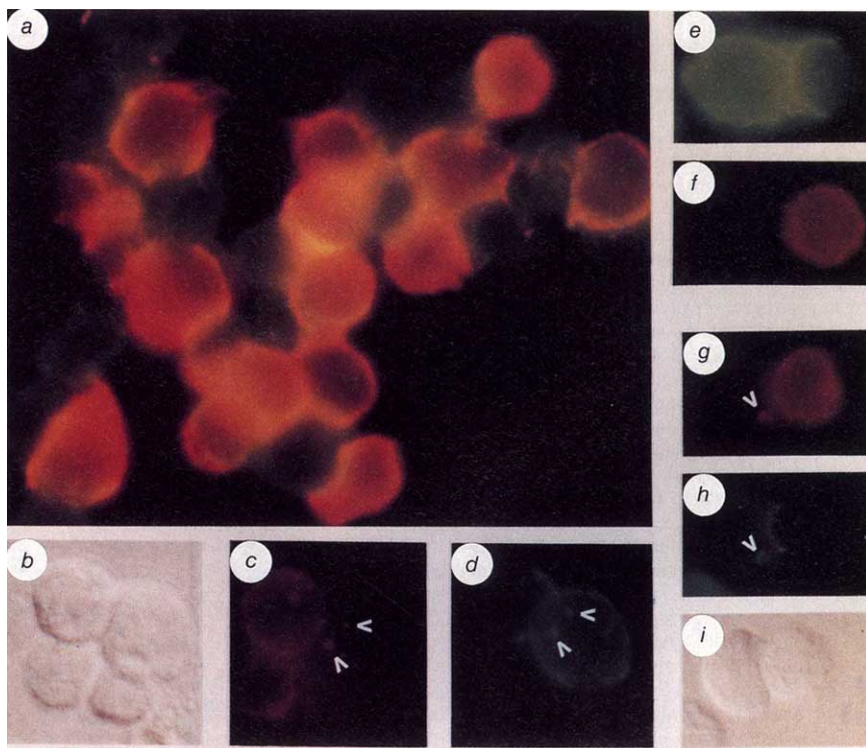
Localization of boss

Further evidence for a direct interaction of boss and *sev* came from immunolocalization studies. In the eye imaginal disk boss is first expressed in cells lying three rows posterior to the morphogenetic furrow (Fig. 4a). Its expression is restricted to the R8 cell, which can be identified by its characteristic shape and position in the developing ommatidial cluster¹² (Figs 4e and 5). A longitudinal section through a stained eye imaginal disk revealed that boss was localized to the apical regions of the monolayer epithelium (Fig. 4c). In this region, boss was highly abundant on the microvillar membrane of the R8 cell (Fig. 5b). Interestingly, *sev* expression is also mainly restricted to the apical membranes of surrounding cells^{19,20}. A cross-sectional view below the microvilli indicated that the entire plasma membrane in the apical region of the R8 cell was stained by the α boss 1 monoclonal antibody (Fig. 5c). Just below this region boss is found in small thin tubule-like structures within the cytoplasm of R8 (data not shown). Further basally, in sections above the nuclei, boss immunoreactivity was observed in multi-vesicular bodies (MVBs) of the R8 cell (Fig. 5d).

Although boss immunoreactivity was not detected on the surface of the R7 precursor cells, it was observed in vesicles within them (Figs 4e and 6). The stained vesicles were most prominently seen in rows 7 to 11 (Fig. 4e). The earliest appearance of boss-containing vesicles in row 6 precedes neuronal differentiation of R7 by 12 hours (see ref. 10 for a description of R-cell differentiation). In R7, boss immunoreactivity was associated with vesicles contained within a larger multi-vesicular body (Fig. 6b). Each R7 precursor cell typically contained a single MVB, although occasionally two were seen. This structure appears to correspond to the MVB in which *sev* can be found in R7 as well as other *sev*-expressing cells (data not shown and ref. 20). A similar morphology occurs for late endosomal compartments in mammalian cells to which peptide ligands such as epidermal growth factor are transported when internalized³². We do not know which part of boss is internalized into the R7

FIG. 3 Immunofluorescence staining of aggregated *boss*- and *sev*-expressing cells. Aggregates of *boss*- and *sev*-expressing cells were stained with the anti-boss and anti-sev antibodies. Boss was visualized (red) using Texas red or rhodamine-labelled secondary antibodies. *Sev* was visualized (green) using FITC-labelled secondary antibodies. a, A cluster of *boss*- and *sev*-expressing cells. b–d and g–i, Intracellular structures in *sev*-expressing cells, presumed to be vesicles (arrowheads), contained both boss and *sev* immunoreactivity. b and i, Clusters visualized by Nomarski optics. e and f, Cells expressing *sev* often appeared to extend their surface over *boss*-expressing cells. One such *sev*-expressing cell is shown in e. Cells were permeabilized in the presence of 0.1% saponin for all panels except e and f which show unpermeabilized cells.

METHODS. Staining of cells was done essentially as described²⁸. In brief, 1 ml of 8% paraformaldehyde in PBS (10 mM sodium phosphate, pH 7.2, 140 mM NaCl, 3 mM KCl) was added to 1 ml of the aggregation mixture, and mixing was continued for 15 min. Cells were collected in a microcentrifuge for 20 s at 10,000 r.p.m., washed twice with BSS and stained for 1 h with affinity-purified rabbit anti-boss antibodies NN-1 (1:3,000) and goat anti-sev serum G15-4 (1:1,000) in BSS¹⁸ adjusted to 0.1% saponin (BSS/saponin). Cells were washed twice in BSS/saponin and stained for 1 h with FITC-conjugated swine anti-goat serum (1:200; Boehringer) which was preabsorbed against rabbit antibodies. After two additional washes in BSS/saponin containing 10% normal goat serum, the cells were stained for 1 h with a rhodamine or Texas red-conjugated goat anti-rabbit antibody diluted with BSS/saponin containing 10% normal goat serum to



1:3,000 or 1:1,000, respectively. After two final washes in BSS/saponin the cells were mounted on slides in PBS containing 90% glycerol and 0.2% phenylenediamine.

precursor cell or the *sev*-expressing S2 cells in the aggregation assay. Experiments using antibodies to specific regions of boss will address this question.

In later stages of eye development *boss* becomes more broadly expressed. In the pupal retina, *boss* was expressed in the bristle neurons, in addition to R8, 24 hours after puparium formation (data not shown). About 24 hours later, after the determination of all the cells in the eye, all photoreceptor cells in the retina expressed *boss* (Fig. 4d). The level of expression in these cells is maintained into adulthood and accounts for the large amounts of *boss* messenger RNA and protein in fly heads (Fig. 1, and ref. 18). Expression of *boss* was also observed in many sensory neurons in the developing antenna and leg disks, in the ocelli (data not shown), and the embryo (D. Van Vactor, unpublished observations). The role of *boss* in the development or physiology of these other cells has yet to be assessed.

Dependence of internalization on *sev*

The boss immunoreactivity found in the MVB of R7 raises the question of whether boss is synthesized in R7 or transferred from R8 into R7. The R8-specificity of the perinuclear ER staining (Fig. 5e) demonstrated that boss is translated only in R8 and not in R7. We used the temperature-sensitive mutation *shibire*^{ts1} (*shi*^{ts1}) to investigate further whether boss immunoreactivity in the R7 precursor cells originates from the internalization of boss protein translated in R8. In *shi*^{ts1} mutants, endocytosis is blocked at the nonpermissive temperature, resulting in an accumulation of coated pits and the loss of intracellular coated vesicles³³. The *shibire* gene (A. van der Blieck and E. Meyerowitz, personal communication) encodes a homologue of rat dynamin³⁴ and yeast Vps1p³⁵, proteins implicated in microtubule-based motility and vesicle movement, respectively. We tested whether boss can be internalized by the R7 precursor cell

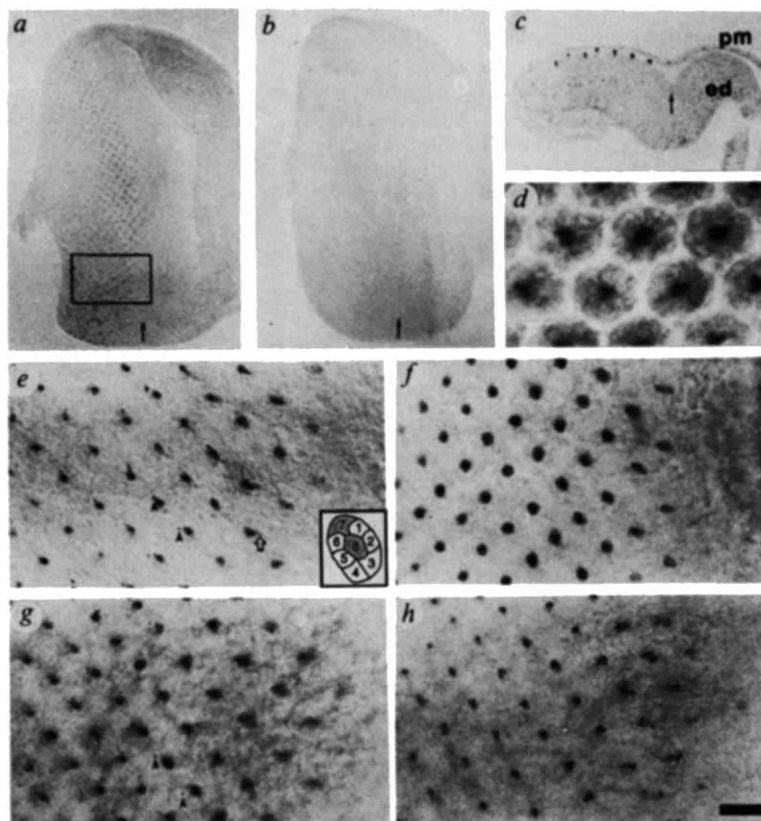
in the *shi*^{ts1} mutant. At the permissive temperature, 123 of 248 R7 precursor cells examined contained boss immunoreactivity in their MVB. This fraction decreased dramatically after 30 min at the nonpermissive temperature (32 °C) to only 9 of 441 cells (Fig. 4h). These observations indicate that boss is endocytosed into R7.

We examined the distribution of boss in the null mutant *sev*^{X1}, which does not express *sev* to find whether boss immunoreactivity in the R7 precursor cell is dependent on the *sev*-encoded receptor. In this mutant, the MVB of the R7 precursor cell lacks boss immunoreactivity (Fig. 4f). This could reflect direct binding of boss to the *sev*-encoded receptor or that boss internalization depends on R7 development and does not occur in a developing cone cell. To distinguish between these two possibilities we used the *sev*^{Met2242} allele which encodes a *sev* protein with a tyrosine-kinase domain in which Lys 2242 is replaced with Met²³. Although this abolishes tyrosine-kinase activity and thus prevents R7 development, the receptor protein is expressed in the same way as wild-type *sev*. Inactivating the tyrosine-kinase activity of other receptors by analogous mutations does not prevent endocytosis of their ligands^{32,36–38}. Similarly, in the *sev*^{Met2242} mutant, boss immunoreactivity was found in the MVB of the R7 precursor cell as it is in wild-type eye disks (Fig. 4g). This demonstrates that the internalization of boss is not dependent on the cell assuming an R7 cell fate and indicates that internalization of boss by the R7 precursor cell is mediated by the binding of boss to *sev*. This provides evidence for a direct interaction between boss and *sev* *in vivo*.

Specificity of internalization

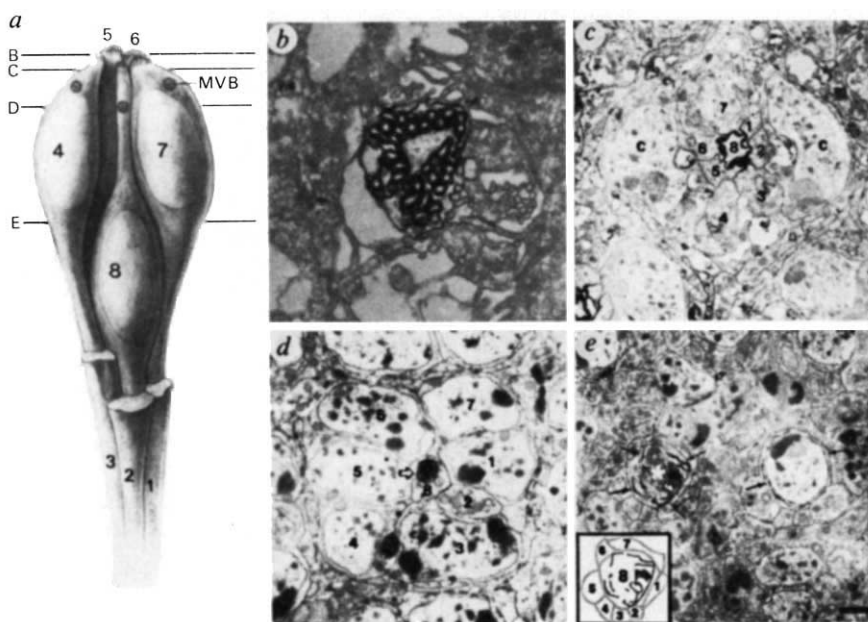
Although boss is internalized by the R7 precursor cell, it is not internalized by other *sev*-expressing cells which contact R8. These cells do contain MVBs, which contain *sev* (ref. 20 and

FIG. 4 Immunolocalization of boss using light microscopy. Whole-mount preparations of eye imaginal discs were stained using α boss1. **a**, In developing wild-type third instar eye disks a single cell in each ommatidium expresses boss. For higher resolution view see Fig. 5. **b**, Staining is not observed in developing *boss*¹ eye imaginal disks. **c**, Longitudinal section through a third instar wild-type eye disk reveals that boss is localized to the apical region of the disk epithelium. In some preparations, faint staining along the entire cell body of R8 could be observed in the most mature (that is, posterior) clusters (data not shown). The arrows in **a–c** indicate the morphogenetic furrow. **d**, All photoreceptor cells are stained in a whole-mount preparation of a wild-type pupal eye disk (48 h post-pupariation). **e–h**, Regions of the disk epithelium that correspond to the rectangle in **a**. **e**, In wild-type flies a small 'satellite' of staining (arrowheads) is visible close to the stained apical region of R8 (see inset). This is due to localization of boss to a multivesicular body (MVB) in the R7 precursor cell (see Fig. 6). In this preparation, staining of MVBs with α boss1 first appears in row seven. Infrequently, stained MVBs can be seen just anterior to R8's apical staining (open arrow). Careful analysis with light and electron microscopy indicated that these MVBs lie within the R8 cell body, which extends anteriorly from its stained apical region. **f**, Loss of the *sev* protein in the *sev*^{X1} mutant does not affect boss expression in R8, but the MVB in the R7 precursor does not contain boss immunoreactivity. **g**, In the *sev*^{Met2242} mutant the tyrosine-kinase activity is inactivated, but protein expression is unaltered²³. In this mutant the R7 precursor cell differentiates as a cone cell²³. The boss protein is localized to both R8, and an MVB in the R7 precursor cell (arrowhead). **h**, Temperature pulses of *shi*^{ts1} flies interfere with internalization of surface membrane³³ and result in the loss of boss immunoreactivity in the MVB of the R7 cell precursor. Temperature-pulsed wild-type and untreated *shi*^{ts1} eye disks exhibited staining indistinguishable from that in **e**. Anterior is to the right, except in **d** where it is towards the top. The scale bar in **h** represents 25 μ m for **a–c**, 10 μ m for **d** and 5 μ m for **e–h**, respectively. Abbreviations: 1–8, photoreceptors R1–R8; ed, eye imaginal disc; pm, peripodial membrane.



METHODS. Localization of boss protein with α boss1 for light microscopy was done as described (D. Van Vactor *et al.*, manuscript in preparation and ref. 20).

FIG. 5 Localization of boss by immunoelectron microscopy. *a*, Cutaway view of a symmetric eight-cell cluster is drawn to scale. The positions along the apical-basal axis of the cross-sectional views presented in the electron micrographs *b–e* are indicated by bars. The nucleus of the R8 cell is positioned more basally than the nuclei of the surrounding cells. The boss protein was detected using α boss1 and visualized by the silver-enhanced HRP method. *b*, The apical microvillar membrane of R8 is strongly stained. *c*, Just below the microvilli, boss is distributed uniformly along the plasma membrane of R8. *d*, About 6 μ m below the microvilli, boss is also found in MVBs in R8 which are similar to those seen in R7 (see Fig. 6). The electron-dense material in the nuclei of the surrounding cells is chromatin. *e*, Basal view of ommatidia near the level of the R8 nucleus, roughly 15 μ m below the microvilli, shows the strong staining of the long thin perinuclear ER of R8 (arrows). A tracing of one ommatidium (asterisk) is shown in the inset. The light lines indicate cell membranes and the dark lines in R8 indicate the stained endoplasmic reticulum. The R8 cell body is larger at this level owing to its more basally localized nucleus. No other photoreceptor in the developing eye disk shows endoplasmic localization of boss (see *d* for a view of other photoreceptor cells at the nuclear level). Anterior is to the right. The scale bar in *e* represents 0.5 μ m for *b*, 1 μ m for *c* and *d*, and 1.5 μ m for *e*, respectively. Abbreviations: 1–8, photoreceptors R1–R8; c, cone cells; ER, endoplasmic reticulum; MVB,



multivesicular body.

METHODS. Antibody staining for electron microscopy was performed as described by D. Van Vactor *et al.* (manuscript in preparation) and ref. 20.

data not shown). This difference seems to be due to the prior commitment of these cells to a different cellular fate (D. Van Vactor *et al.*, manuscript in preparation). The internalization of boss *per se* is not necessary for R7 induction as demonstrated by a boss-independent gain-of-function allele of *sev*³⁹. But the restriction of boss internalization to the only cell to respond to the R7-inductive signal raises the possibility that the internalization of the activated *sev*-encoded receptor is an essential step in the transduction of this signal.

The differential ability of cells to internalize boss could be due to a difference in the binding between boss and *sev*. Alternatively, internalization itself could be regulated. Mechanisms that establish differential competence to respond to a signal in other developmental systems may have a role here as well. For example, the differential response of dorsal and ventral ectoderm to neuronal induction during embryonic development of *Xenopus*⁴⁰ has been attributed to the differential expression of protein kinase C isozymes⁴¹. Phosphorylation by protein kinase C can change the affinities of tyrosine-kinase receptors for their

ligands and their kinase activity^{42,43}. Alternatively, the addition or removal of terminal sugars on *O*- or *N*-linked carbohydrates is an important mechanism regulating several different cell-cell interactions⁴⁴.

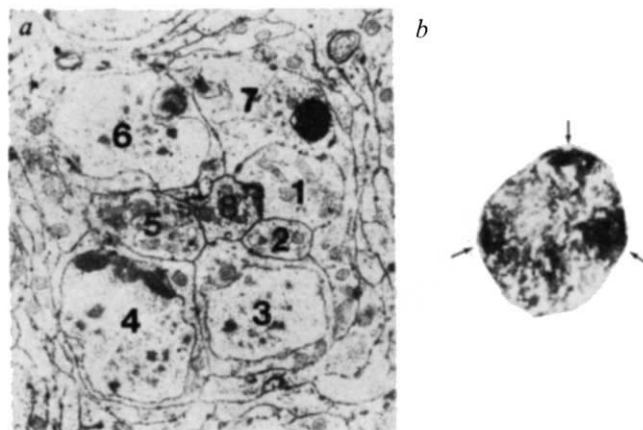
Concluding remarks

Genetic mosaic analyses of the *boss* gene provided the first evidence that an inductive cue from the R8 cell was required for R7 development¹⁴. It further suggested that *boss* encodes the inductive cue received by the R7 precursor cell through the *sev*-encoded tyrosine-kinase receptor. We have presented *in vivo* and *in vitro* evidence supporting this hypothesis. First, boss is expressed exclusively in R8 and is internalized in a *sev*-dependent manner by R7. And second, *boss*- and *sev*-expressing S2 cells form heterotypic aggregates.

The proposed structure of boss, containing a large extracellular domain and seven putative transmembrane-spanning segments, is novel for a ligand of a tyrosine-kinase receptor. It will be interesting to see whether other tyrosine-kinase receptors

FIG. 6 The boss-containing MVB in the R7 precursor cell. The boss protein was detected using α boss1 and visualized by the silver-enhanced HRP method and electron microscopy. *a*, A section 5 μ m below the apical surface shows a single ommatidium at the symmetrical eight cell cluster stage⁵⁵. This plane of section is just below the apical region where boss is localized to the R8 plasma membrane; a small amount of staining can still be seen juxtaposing R1 and R6. R7 contains an intensely stained MVB similar to that seen in R8 (Fig. 5*d*). The absence of staining in the perinuclear ER of R7 (see Fig. 5*e*) indicates that boss is not made in R7. *b*, An enlarged view of a MVB similar to the one seen in *a*. The MVBs found in R7 are membrane-bound vesicular structures rich in internal membranes similar to those described for localization of other ligand-receptor complexes³². Boss is localized to discrete regions within this MVB (arrows). Anterior is to the right. The scale bar in *b* represents 1 μ m for *a* and 0.3 μ m for *b*, respectively. Abbreviations: 1–8, photoreceptors R1–R8.

METHODS. Antibody staining for electron microscopy was performed as described by D. Van Vactor *et al.* (manuscript in preparation) and ref. 20.



with so far unknown ligands bind to proteins with a similar structure. The binding of a membrane-anchored ligand to a tyrosine-kinase receptor is not without precedent. The membrane-anchored precursor proteins of peptide hormones such as transforming growth factor α or *kit* ligand growth factor can elicit cellular responses on binding to specific receptors⁴⁵⁻⁴⁷ and mediate cell-cell adhesion^{47,48}. The anchoring of a ligand to a cell surface provides a way in which an inductive cue can be restricted to only cells that contact the inducing cell. The functional importance of tethering boss to the R8 membrane is indicated by experiments involving ectopic expression of *boss*, in which *sev*-expressing cells not in contact with R8 become R7 cells (D. Van Vactor *et al.*, manuscript in preparation).

We do not know whether the seven transmembrane domains of boss directly participate in binding to *sev* or whether they serve any other function. Proteins with similar overall structure such as the receptors for thyrotropin⁴⁹ or luteinizing hormone/chorionic gonadotropin^{50,51} function in the reception of a signal rather than as a ligand. It remains possible that boss acts as both a ligand and a receptor. Indeed, during cell-cell

signalling mediated by two such interacting transmembrane proteins, each might function as both a ligand and a receptor. But the R8 cell seems to develop normally in a *boss* mutant¹⁸, so as yet there is not any evidence that boss acts as a receptor during R8 development. The expression of boss in a variety of sensory neurons throughout the life-cycle of *Drosophila* in regions where *sev* is not expressed raises the possibility that boss has a function in addition to its role in R7 development.

In summary, boss is a membrane-anchored ligand that binds to the *sev*-encoded receptor to induce R7 cell fate. The precise regulation of the time and place of boss expression, together with the cell-surface localization of its protein provides a way for restricting an inductive cue to only the group of cells that contact the inducing cell. The restriction of a cell's ability to respond to an inductive cue may also be regulated by the developmental state of the responding cell. It is attractive to speculate that similar strategies are used in other systems where local cell-cell interactions are thought to regulate the assembly of various cell types into precisely arranged cellular networks. □

Received 9 May; accepted 19 June 1991.

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ACKNOWLEDGEMENTS. We thank A. Hart and D. Van Vactor for discussions; U. Banerjee, S. Crews, E. DeRobertis, G. Payne and O. Witte for criticisms of the manuscript; B. Pilz and D. Meyer for technical assistance in the production of the monoclonal antibodies; J. Young and the UCLA peptide synthesis facility for peptides; U. Banerjee and S. Benzer for the antibody 150C3; E. Hafen for the goat anti-*sev* antibodies and the *sev*^{Met2242} fly stock; M. Simon, D. Bowtell and G. Rubin for the SL2-*sev* cell line and the *hs/cDNA*^{sev} DNA; F. Eisinger and G. Zampighi for the use of electron microscopes and ultramicrotomes; S. Crews and U. Banerjee for the use of their Axiophot microscopes, and S. Belkin for artwork. This work was supported by an EMBO postdoctoral fellowship (H.K.) a postdoctoral fellowship from the NIH (R.C.), a Faculty Research Award from the American Cancer Society (S.L.Z.), an award from the McKnight Foundation (S.L.Z.) and an NIH grant.

Structural basis of anticodon loop recognition by glutamyl-tRNA synthetase

M. A. Rould, J. J. Perona & T. A. Steitz

Departments of Molecular Biophysics and Biochemistry, and Chemistry, and Howard Hughes Medical Institute, Yale University, PO Box 6666, New Haven, Connecticut 06511, USA

The refined crystal structure of *Escherichia coli* glutamyl transfer RNA synthetase complexed with transfer RNA^{Gln} and ATP reveals that the structure of the anticodon loop of the enzyme-bound tRNA^{Gln} differs extensively from that of the known crystal structures of uncomplexed tRNA molecules. The anticodon stem is extended by two non-Watson-Crick base pairs, leaving the three anticodon bases unpaired and splayed out to bind snugly into three separate complementary pockets in the protein. These interactions suggest that the entire anticodon loop provides essential sites for glutamyl tRNA synthetase discrimination among tRNA molecules.

THE aminoacyl-tRNA synthetases can be grouped into those that use the anticodon of the tRNA to discriminate between cognate and non-cognate species, and those that do not¹⁻³. Genetics and biochemistry show that glutamyl-tRNA synthetase (GlnRS) from *E. coli* does use the anticodon for this discrimination. A mutation of the central anticodon base from C to U converts *E. coli* tRNA^{Trp} into a tRNA that alleviates the effects of amber mutations (an amber suppressor) and that is acylated equally well by GlnRS as by TrpRS^{4,5}. By enzymatically excising the anticodon from initiator tRNA^{Met} (CAU) and inserting the anticodon for an amber suppressor (CUA), Schulman and Pelka¹ generated a hybrid tRNA that was a less efficient substrate for MetRS but could be charged by GlnRS 1,000 times faster than tRNA^{Met}. Uracil at position 35, the central anticodon nucleotide, is thus a main identity determinant of tRNA^{Gln}.

The results of these and related studies, however, were interpreted to mean that the anticodon nucleotide 36 is not involved in recognition by GlnRS, because the suppressor tRNAs, with an A at this position instead of the G in tRNA^{Gln}, could be charged *in vitro* with glutamine and could suppress termination *in vivo*. Because GlnRS normally charges two isoaccepting tRNAs that have either a C or 2-thio-U at position 34, stringent recognition of this nucleotide was not expected.

Previous crystallographic analysis of the GlnRS-tRNA complex at 2.8 Å resolution allowed unambiguous interpretation of electron density maps for most of the enzyme and tRNA structure and implicated seven nucleotides in the acceptor stem and 3' strand as potential tRNA^{Gln} identity elements⁶. Although it was clear earlier that the anticodon region interacts with the protein, determination of the detailed structure of the complex near the anticodon was hindered by poor electron density for this region. The co-crystal structure of the GlnRS-tRNA complex refined at 2.5 Å resolution reported here implies that all three anticodon bases contribute significantly to tRNA discrimination by GlnRS. In addition it seems that the other four bases of the anticodon loop also serve as tRNA^{Gln} identity elements

by virtue of their interactions with the protein and formation of two non-Watson-Crick base pairs. With the seven nucleotides in the acceptor stem and 3' strand described earlier, at least 14 nucleotides are implicated by the structure to be identity elements.

Refinement at 2.5 Å resolution

We have established the detailed structure of the protein and tRNA near the anticodon by cooling crystals in the presence of glycerol. A vastly improved density map has been obtained using new X-ray diffraction data collected to 2.5 Å resolution from crystals cooled to -8 °C in the presence of 20% (w/v) glycerol. Amino-acid side chains in the anticodon were identifiable in the new electron density map calculated at 2.5 Å resolution using $2F_o - F_c$ as Fourier coefficients, which has elucidated the structure of the interface between the protein and the tRNA in the anticodon region (Fig. 1). We do not know whether the glycerol or lower temperature, or both, are responsible for the increased order of the two β-barrel domains interacting with the anticodon (Fig. 2).

Conformational differences in anticodon loop

Although the anticodon stem and loop of free tRNAs whose crystal structures have been determined (9-13) display a similar conformation, the structure of this region of the enzyme-bound tRNA^{Gln} differs substantially from that of the unbound tRNAs (Fig. 3a). In tRNA^{Asp}, tRNA^{Met} and tRNA^{Phe}, the three anticodon bases stack on one another, projecting their Watson-Crick moieties outward. The region of the anticodon loop nearest the stem is stabilized by a hydrated magnesium ion in both cases; no such ion is seen in the enzyme-bound tRNA^{Gln}. The anticodon stem of complexed tRNA^{Gln} is extended by two non-Watson-Crick base pairs, leaving the bases of the anticodon splayed outwards in different directions enabling them to interact with separate recognition pockets in the protein. On the basis of NMR experiments, Redfield and co-workers¹⁴ suggested that the environment of the anticodon region of tRNA^{Gln} changed on binding GlnRS, consistent with our expectation that the free tRNA^{Gln} structure resembles the other unbound tRNAs and changes on forming a complex.

Formation of two novel non-Watson-Crick base pairs between U and pseudo-U and between A and U extends the RNA helix of the anticodon stem. There is a single direct hydrogen bond between bases 2'-O-methyl-U32 and pseudo-U 38 and between U33 and 2-methyl-A37 in the anticodon loop (Fig. 3b, c). These bases stack with the anticodon stem. This extension of the anticodon stem is further stabilized by a water-mediated hydrogen-bonding network between the bases (including the N1 nitrogen of pseudo-U), the sugar-phosphate backbone and the protein. The sugar puckers of nucleotides 32, 33, 37 and 38 of the anticodon loop are C3'-endo as expected in A-form helical RNA, whereas the pucker of the three anticodon nucleotides is C2'-endo. The solution structure of an RNA stem and loop of different sequence established by NMR¹⁵ also shows a stem region extended by three non-Watson-Crick base pairs, leaving only the final three bases unpaired.

Anticodon loop in tRNA discrimination

It seems from the GlnRS-tRNA^{Gln} structure that all seven bases of the anticodon loop are used by GlnRS to discriminate among tRNAs. The non-anticodon bases in the loop may be able to serve as identity elements because of: their ability to assume a structure that is essential for tRNA binding; the direct interactions they make with the protein; and bulky modifications that occur at nucleotides 34 and 37 of many non-cognate tRNAs.

Direct recognition of bases 37 and 38 is achieved by the side chain of Asn 370, which forms hydrogen bonds with the bases of pseudo-U 38 and 2-methyl-A 37 in the minor groove of the tRNA (Fig. 3*b, c*). The side chains of Thr 547 and Arg 545 bind to the backbone phosphates of residues 37 and 38, interactions that require the formation of the extended anticodon stem and thus may be indirectly important for discrimination. Changing any of the four bases of the extended stem affects aminoacylation of suppressor tRNAs by GlnRS¹⁶.

Base modifications of non-cognate tRNAs at nucleotides 34 and 37 may act as negative determinants of aminoacylation by GlnRS. The tightly packed interface between A37 and the protein (Figs 3, 4) suggests that bases with bulky modifications of A37, to give, for example, 6-carbamoyl-threonyl adenine or 2-methylthio-N6-isopentenyl adenine, may provide an additional source of discrimination against the many non-cognate tRNA molecules bearing these modifications. A role for modified bases at position 37 in tRNA discrimination has already been suggested by biochemical studies showing that ArgRS misacylates tRNA^{Asp} lacking modified bases¹⁷. Likewise, C34 is tightly packed into a pocket that is covered by a loop of protein (Figs 4, 5); this pocket may not accommodate certain modified bases at position 34, such as queuosine.

The three anticodon nucleotides adopt unusual conformations with each of the bases projecting into protein pockets that are complementary to the two glutamine anticodons.

C34 binding pocket. Because there are two isoaccepting tRNA species that GlnRS must recognize, one with anticodon CUG (as in the co-crystal) and the other with anticodon UUG, the recognition pocket for nucleotide 34 must be able to accommodate either pyrimidine, and perhaps discriminate against purines. The Watson-Crick hydrogen-bonding groups of C34 are specifically recognized by a peptide carbonyl oxygen, a peptide amide and a guanidinium group that also anchors the backbone phosphate (Fig. 5*a*).

How might this pocket accommodate both pyrimidines and discriminate against purines? Clearly the 2-thio-U at position 34 of the isoacceptor tRNA^{Gln} cannot bind in an identical manner to this enzyme pocket. But with a small shift in the base, the 4-keto group can be modelled to interact with the guanidinium of Arg 410 instead of the carbonyl of 414, the larger 2-thio group being bound the same way as the 2-keto of C34. We anticipate, however, that purines are sterically excluded from the pocket. Residues 443–453 (between strands 18 and 19), which are partially disordered, pass over nucleotide 34, forming a floppy lid to the binding-site loop (Fig. 4). This lid on the nucleotide 34 pocket may provide additional discrimination against frequently occurring¹⁹ large modified bases such as queuosine. Consistent with this proposal is the observation that cyanylation of the 2-thio-U of tRNA^{Gln} results in a 10-fold reduction in the rate of glutaminylation¹⁸.

U35 binding pocket. Of the three anticodon bases, U35 has the tightest binding pocket. Arginines 412 and 520 and lysine 401 anchor this nucleotide into its binding pocket through ionic interactions with its two adjacent phosphates; the aliphatic portion of Arg 520 packs against the ribose of residue 35. A hydrogen bond between the 5' phosphate of residue 35 and the sugar 02' of residue 33 helps to stabilize a conformation of the tRNA backbone that allows the uracil base to stack under the 2-methyl-adenine of 37; Pro 369 packs against the other face of U35. These interactions present the base of nucleotide 35 onto a recognition surface that is complementary to the Watson-Crick

moieties of a uracil (Fig. 5*b*). Recognition is achieved through buried hydrogen-bonding interactions with Arg 341, Gln 517, a peptide amide and perhaps Glu 519. A total of four basic and one acidic amino-acid side chains interact with this one nucleotide.

G36 binding pocket. Guanine 36 binds into a site that is complementary to guanosine and not the other three bases (if the protein structure remains fixed) making G36 a likely recognition element (Fig. 5*c*). Recognition of G36 by the enzyme was not anticipated, because amber suppressor tRNAs containing an A at this position are glutaminylated sufficiently to suppress a premature termination codon in *lacZ*1000 (ref. 20). An important recognition interaction is between the guanidinium group of Arg 402 and N7 and 6-keto groups of guanine. Although a clear fitting of the C-terminal six amino acids of the enzyme has not been possible, density in this region suggests that part of it packs against the purine sandwiching it against Lys 401.

Similarities among pockets

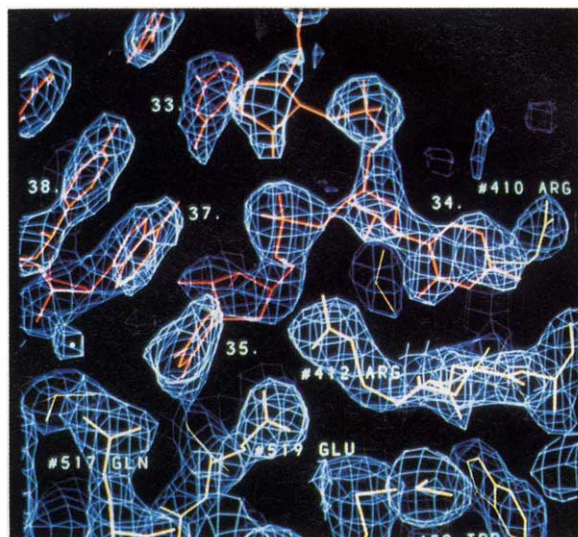
Each anticodon nucleotide is recognized primarily by a polypeptide segment of five or six amino acids. In all three cases, at least one positively charged amino acid from this segment forms a salt-link with an adjacent negatively charged phosphate. The aliphatic portion of this residue generally packs against either the base or the hydrophobic 'underside' of ribose. With all three anticodon bases, recognition is achieved by direct hydrogen-bonding between the backbone and side chains of a short recognition peptide and the Watson-Crick hydrogen-bonding groups of the bases. Further, several of the interactions presumed to be discriminating involve hydrogen-bonds with charged side chains that are buried from solvent in the complex. Although there is no conserved sequence or structural similarity among these segments, they are predominantly in extended β -type conformation. It is interesting to note that the ATP in the active site is also recognized by a similar polypeptide segment (J.J.P., M.A.R. & T.A.S., manuscript in preparation).

Allosteric linkage of anticodon and active site?

Although transcription regulator proteins discriminate among potential DNA-binding sites entirely on the basis of dissociation constants, the same is not true for aminoacyl-tRNA synthetases. Kinetic parameters for misaminoacylation of anticodon modified tRNA^{Met} (ref. 1) as well as for acylation of tRNA^{Gln} containing anticodon mutations²¹ show that although the Michaelis constant K_m is increased for tRNAs with incorrect anticodons, most of the reduced acylation rate results from a decreased catalytic constant k_{cat} . This could result from the active site of the enzyme having a different configuration or the tRNA being bound differently for correct and incorrect anticodons. Because the anticodon is more than 35 Å away from the active site, the signal that the correct anticodon is bound must be transmitted either through the enzyme, the tRNA, or some combination of both.

The two β -barrel domains that bind the anticodon stem and loop are covalently connected to the rest of the protein by an α helix and non-covalently interact through an interface that is primarily hydrophobic. These two domains have higher mobility than the rest of the molecule, as indicated by the higher B -factors. Extending from the junction of the two β barrels is a long two-stranded β ribbon (residues 462–502) that forms part of the proximal β barrel and packs against portions of the two highly conserved peptides that provide part of the ATP-binding site (Fig. 6). The HIGH (residues 40–43; His-Ileu-Gly-His) and MSK (268–270; Met-Ser-Lys) sequences that are highly conserved among, and serve to define, the class I synthetases²² interact with each other and with the phosphates of ATP, presumably having a role in catalysis. Although the loop at the end of the β ribbon does not directly contact these three residues, it does form an intimate interface with the flanking residues. In the other two synthetases whose structures in the absence of tRNA

FIG. 1 A portion of an 'omit map' in the anticodon region. This map was calculated using phases derived from a model with the atoms shown in the figure omitted. The complex was previously refined with cycles of conventional crystallographic coordinate refinement with XPLOR⁷, and manual rebuilding assisted by FRAGLE⁸ brought the *R*-factor for the model (including 227 well-ordered water molecules) to 21.0% for all data in the resolution range 6–2.5 Å (37,820 unique reflexions), with good stereochemistry (r.m.s. bond deviations from ideality: 0.008 Å in the protein, 0.009 Å in the tRNA; r.m.s. angle deviations: 2.3 degrees in the protein, 3.2 degrees in the tRNA). Still disordered are the N-terminal seven amino acids, the C-terminal six amino acids, which pack against G36, and eleven residues from 443 to 453, some of which pack on top of C34. The coordinates will be deposited in the Brookhaven Data Bank.



and co-crystallized ATP are known, MetRS²³ and TyrRS²⁴, these three residues are not well ordered and have a different conformation. The GlnRS structure suggests that the MSK sequence is well ordered in this enzyme and interacts with the ATP partly as a result of the β ribbon properly packing on top of it.

How then might the signal that the correct anticodon is bound be transmitted to the active site? We suggest that in the absence of tRNA or in the presence of a tRNA with the wrong anticodon the two β -barrel domains and the long anti-parallel β arm have a different disposition relative to the rest of the enzyme, and perhaps are even disordered. Further, in the absence of interactions with the β arm, the highly conserved sequences (HIGH and MSK) that form part of the binding site for the phosphates of ATP could also have a different conformation. Incorrect orientation of the ATP phosphates could account for the inability of this enzyme to catalyse the formation of Gln-AMP²⁵, the

first step in the reaction, in the absence of tRNA. We expect that only in the presence of the correct anticodon is the proper binding site of the triphosphate moiety of ATP formed.

Other identity elements

In addition to the seven nucleotides in the anticodon loop of tRNA^{Gln}, the co-crystal structure implicates as many as nine other nucleotides located in the acceptor stem and D stem and loop whose identities may affect the charging of this tRNA by GlnRS (Fig. 7). Direct sequence-specific contact between the enzyme and the 2-amino groups of G2 and G3 in the minor groove of the acceptor stem⁶ and G10 of the D stem implicate these bases as determinants of tRNA^{Gln} identity. A well-ordered water network between the minor grooves and the protein enforces the requirement for base pairs at positions G2-C71, G3-C70 and G10-C25. The enzyme breaks the terminal base

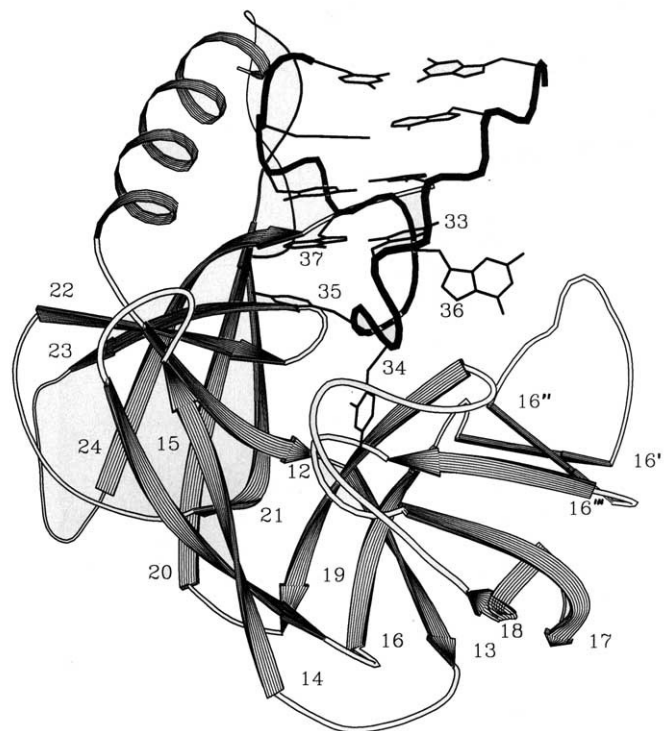


FIG. 2 A schematic drawing (program RIBBON)²⁶ of the two β -barrel domains of GlnRS that interact with the anticodon stem and loop. Beta strands (represented by arrows) 12 and 20–24 comprise the proximal β barrel; strands 13–19 comprise the distal β barrel. The base of C34 is recognized by residues of the loop between strands 16'' and 17; U35 by residues of strand 22 and Arg 341 of strand 12; G36 by the loop between strands 16' and 16''. Interactions with bases 37 and 38 are made by the loop between strands 14 and 15. The loop between β strands 20 and 21 packs against the active-site domain. The amino-acid residue numbers corresponding to the structural elements are: β 12, 340–346; β 13, 347–353; β 14, 360–367; β 15, 375–382; β 16, 384–389; β 16', 391–394; β 16'', 402–405; β 16''', 407–411; β 17, 415–425; β 18, 429–438; β 19, 454–461; β 20, 465–473; β 21, 495–504; β 22, 514–519; β 23, 521–526; β 24, 536–544.

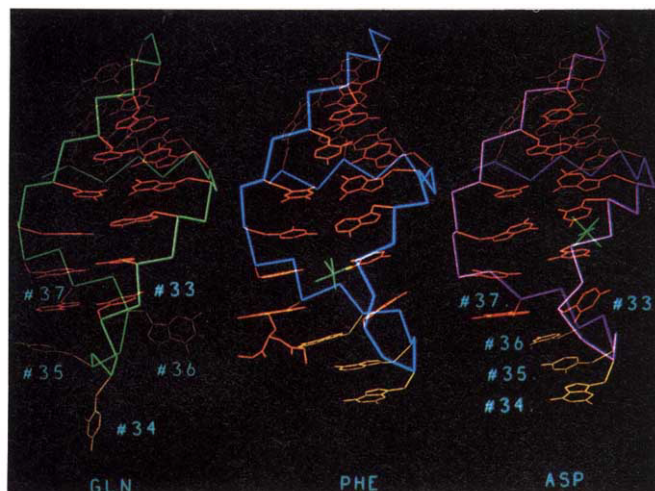


FIG. 3 *a*, Comparison of the anticodon stem and loop structure of enzyme-bound *E. coli* tRNA^{Gln} with those of unbound tRNA^{Phe} and tRNA^{ASP} from yeast viewed from the same orientation of the anticodon stem as in Figs 2 and 4. The sugar-phosphate backbone is schematically represented by the positions of the phosphorous and C4' atoms. Whereas the anticodon bases of the unbound tRNA's stack on each other, these bases splay outwards in the complex to interact with binding pockets in the protein. In the bound tRNA, the anticodon stem is extended by two non-Watson-Crick base pairs (32–38 and 33–37), a condition essentially precluded in the unbound tRNA's by the presence of a hydrated magnesium ion. tRNA^{Phe} contains the hypermodified base wybutosine at position 37. *b*, A hydrogen-bond is seen between atom N3 of 2'-O-methyl-U32 and atom O2 of pseudouridine 38 to form a non-Watson-Crick U-U base pair. Water molecules, shown as dark balls, form additional stabilizing hydrogen bonded networks. *c*, A hydrogen-bond is seen between atom O4 of U33 and the 6-amino nitrogen of 2-methyl-A37 to form a second non-Watson-Crick U·A base pair that is also stabilized by interactions with bound water. Sequence-specific interactions are made between Asn 370 and 2-methyl-A37 and pseudo-U38.

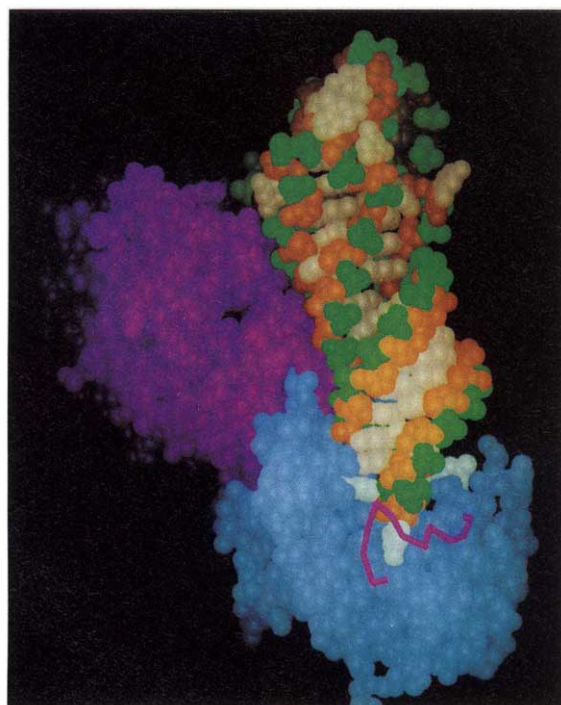
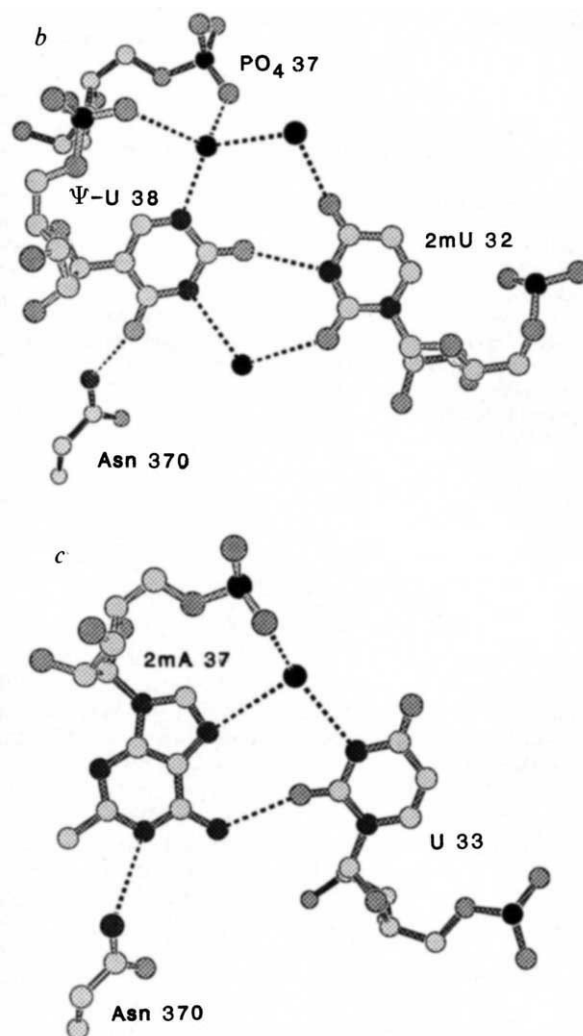


FIG. 4 Space-filling model of the GlnRS-tRNA complex, with the anticodon recognition domains in blue and the N-terminal domains in red, oriented as in Figs 1 and 2, with the minor groove of the anticodon stem facing forward. Each anticodon base (white) binds into a separate pocket in the enzyme. Loosely packing across C34 are residues 443–453 (shown as a C α backbone in red), which are partially disordered in the co-crystal structure and thus form a floppy lid to the binding pocket. U35 packs under 2-methyl-A37 and the 3' stack of bases. The tight interface between the enzyme and the minor groove in the region of nucleotides 37 and 38 suggests that GlnRS would sterically discriminate against non-cognate tRNAs with bulky modifications of the base of 37. Likewise, bulky modifications of the base at nucleotides 34 would also be sterically excluded from the binding pocket.

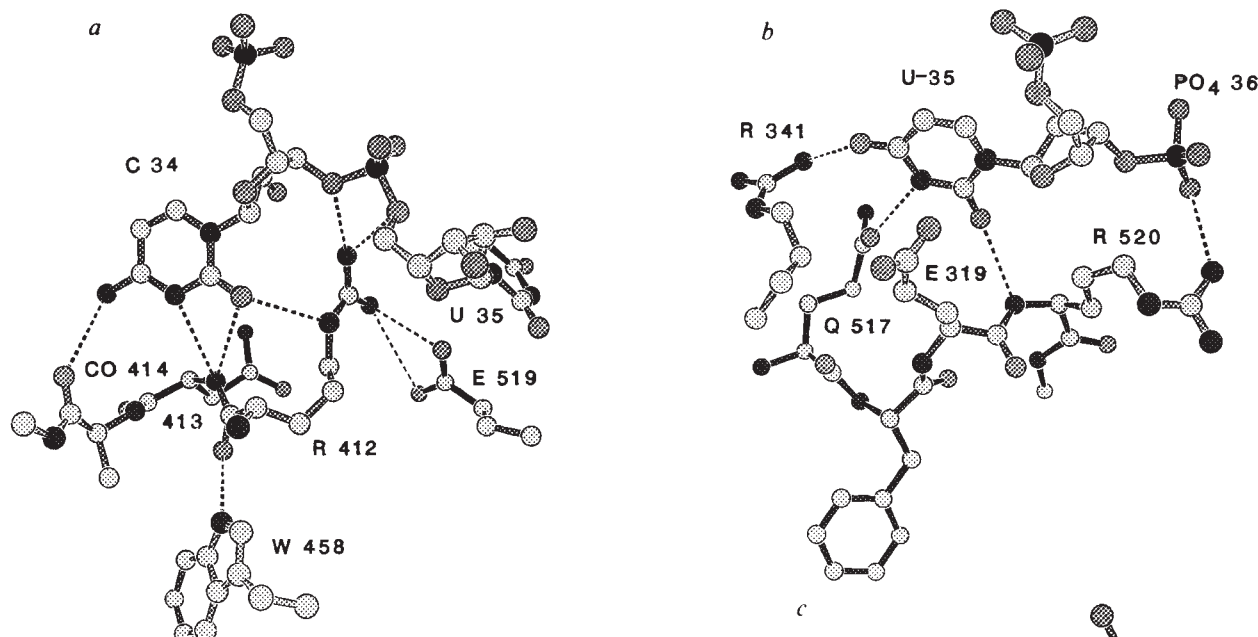


FIG. 5 Ball and stick models of each of the anticodon nucleotides interacting with the protein. Hydrogen bonds are represented by dashed lines. Direct recognition of the anticodon nucleotides is accomplished largely by a single short polypeptide in each case. *a*, Specific C34 interactions. Arg 412 of this loop serves the dual purpose of pulling the nucleotide into its recognition pocket by its 3' phosphate and interacting with the exocyclic 2-keto group of the pyrimidine. Hydrogen-bonded to the same keto oxygen is the amide NH of the peptide linkage between residues 412 and 413 which in turn is oriented by interaction of its carbonyl with the ring nitrogen of Trp 458. This amide NH makes a bifurcated hydrogen-bond to the ring N3 hydrogen-bond acceptor of C34. The exocyclic 4-amino group of the cytosine is also seen to interact with peptide backbone, the carbonyl oxygen between residues 414 and 415. Forming one wall of the C34 binding pocket is the guanidinium moiety of Arg 410 (not shown). *b*, Specific U35 interactions: (1) the O2 of the uracil forms a strong hydrogen-bond with the backbone NH of Arg 520; (2) The N3 hydrogen-bond donor of the uracil sits on top of the amide side chain of Gln 517; (3) the exocyclic O4 contacts the guanidinium ion of Arg 341, which itself is tightly held in place by two backbone carbonyl oxygens at the C terminus of a long α helix. Pointing into the flat face of the uracil is the carboxylate side chain of Glu 519. *c*, Specific G36 interactions. Lys 401 extends from this segment to pull the nucleotide into its binding pocket by its 5' phosphate and the aliphatic portion of its side chain packs against the aromatic rings of the base. The guanidinium group of Arg 402 forms a positively charged cradle onto which the ring N7 and exocyclic 6-keto groups rest. A backbone peptide carbonyl oxygen from residue 399 hydrogen-bonds to both the N1 ring nitrogen and the 2-amino group. Not directly interacting with the base, Tyr 400 is mainly responsible for maintaining the conformation of this loop so that the carbonyl oxygen of 399 is rigidly directed toward the base.

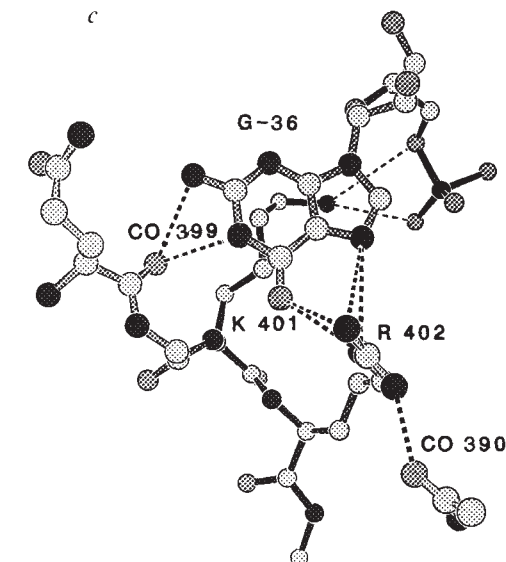
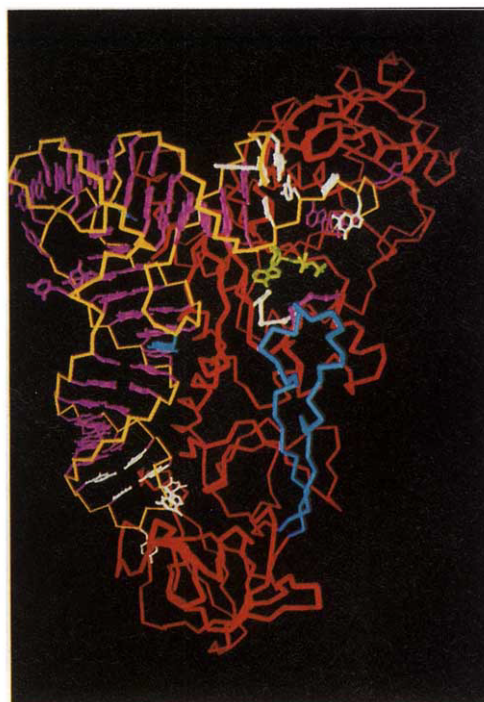


FIG. 6 Nucleotides suggested by the structure as identity elements of tRNA^{Gln} (white bases: 1, 2, 3, 32, 33, 34, 35, 36, 37, 38, 70, 71, 72, 73), and bases seen to interact with the enzyme but whose role in tRNA identity is less certain (blue bases: 10, 16). A long two-stranded β ribbon extends from the junction of the two β -barrel domains that interact with the anticodon, to the active-site domain. The two sequences characteristic of class I synthetases are indicated (purple: M268, S269, K270; white: H40, I41, G42, H43). The side chain of Lys 270 interacts with the phosphates of the ATP (green).



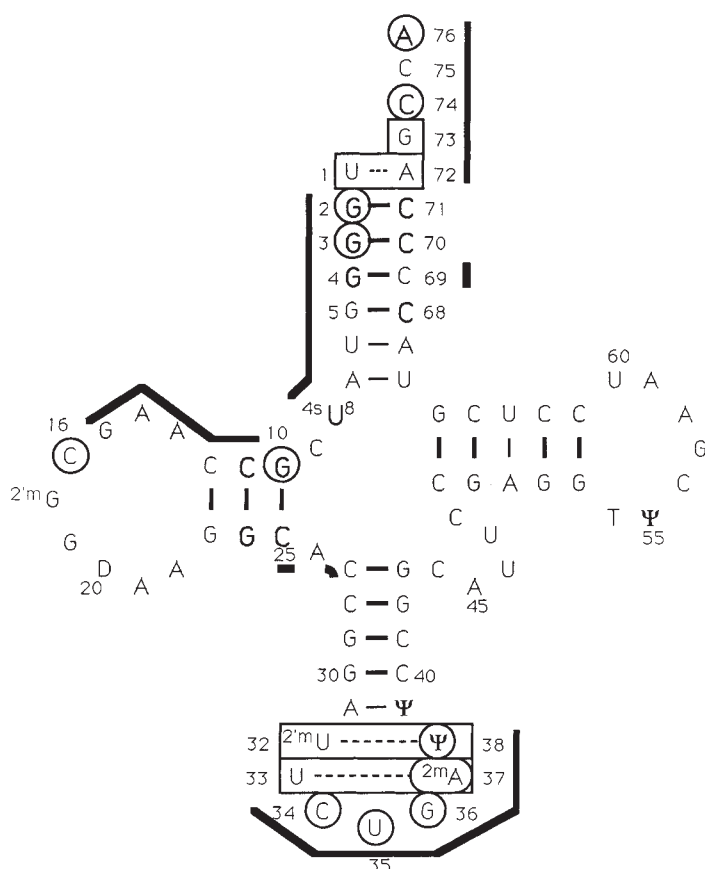


FIG. 7 A schematic summary of protein-RNA and RNA-RNA interactions seen in the refined GlnRS-tRNA crystal structure. Bases directly and specifically recognized by the enzyme are circled, whereas bases that interact with the protein through a single water molecule are in bold. Nucleotides that facilitate (in a base-specific manner) the tRNA in assuming the conformation necessary for its binding to the protein are boxed. Interactions between the enzyme and sugar-phosphate backbone are indicated by thick dark lines. Thus, the nucleotides that seem from the co-crystal structure to be important for tRNA discrimination by GlnRS are: G73, U1·A72, G2·C71, G3·C70, G10, C16, 2'-methyl-U32-, pseudo-U38, U33·2-methyl-A37, C34, U35, G36.

pair of the acceptor helix⁶, and thereby discriminates against the many tRNA's with the more stable G·C base pair here¹⁹. The discriminator base G73 assists in stabilizing the hairpin conformation of the 3' strand of the tRNA through a strong hydrogen bond formed between its 2-amino group and the phosphate backbone⁶. Base-specific interactions between C16, a phosphate of the tRNA, and the enzyme suggest this base may also play a part in tRNA^{Gln} identity (data not shown).

Conclusions

There are at least three general ways in which nucleotides function as identity elements for recognition by GlnRS: (1) through direct and water-mediated contacts with the protein in the minor groove (G2·C71, G3·C70, G10·C25); (2) through their

ability to form RNA structures required for tRNA binding that other nucleotides do not favour (G73, U1·A72, pseudo-U38·2'-O-Methyl-U32, 2-Methyl-A37·U33); and (3) through single-stranded bases binding in specific 'recognition' pockets on the enzyme (C34, U35, G36, C16). Although detailed structural complementarity is a key part of identity, it is expressed kinetically in k_{cat} possibly through tRNA-induced changes in protein conformation. What is the source of the energy for protein and tRNA conformational changes? About 2,700 Å² of protein surface is buried on formation of the tRNA complex, providing many protein-tRNA interactions to compensate for the reduction in the flexibility of the two macromolecules and for the alteration of their structures. □

Received 8 April; accepted 21 June 1991.

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ACKNOWLEDGEMENTS. We thank D. Söll and coworkers for communicating their results on the kinetics of mutant tRNA^{Gln} molecules before publication. This research was supported by the NIH.

Discovery of ten millisecond pulsars in the globular cluster 47 Tucanae

R. N. Manchester*, A. G. Lyne†, C. Robinson†, N. D'Amico‡, M. Bailes† & J. Lim§

* Australia Telescope National Facility, CSIRO, Epping, New South Wales, 2121, Australia

† University of Manchester, Nuffield Radio Astronomy Laboratories, Jodrell Bank, Macclesfield SK11 9DL, UK

‡ Istituto di Fisica dell'Università di Palermo and Istituto di Radioastronomia del CNR, Bologna, Italy

§ School of Mathematics, Physics, Computing and Electronics, Macquarie University, North Ryde, New South Wales, Australia

IN the past four years a total of 13 millisecond pulsars have been found in 12 different globular clusters. These pulsars are believed to be old neutron stars that have been spun up ('recycled') in low-mass X-ray binary systems¹, although some may have been formed by the accretion-induced collapse of white dwarfs in binaries². The globular cluster 47 Tucanae has an especially dense core, and is therefore a likely site for millisecond pulsar formation. Using the Parkes radiotelescope, we have now detected ten additional millisecond pulsars in 47 Tuc, more than half of which are members of binary systems. Almost half of the known millisecond pulsars and more than a quarter of the known binary pulsars now reside in this one cluster.

47 Tuc is a particularly good candidate for searches for millisecond pulsars. As the core is massive and dense, there is a high probability that accreting binary systems will form, either by tidal capture of solitary neutron stars by normal stars or by exchange interactions with primordial binary systems. Furthermore, its estimated distance³ of 4.1 kpc is only about half the distance to most galactic globular clusters, making detection of low-luminosity millisecond pulsars less difficult.

A 5.75-ms pulsar in 47 Tuc, which we identify as PSR0021–72C, was detected⁴ from data obtained at the Parkes radiotelescope at a wavelength of 50 cm between July and December 1989. Further analysis of these data, together with analysis of new data recorded at the Parkes radiotelescope up to February 1991, has revealed a further ten millisecond pulsars in the direction of the cluster. The data set consists of 80 observations, most of duration either 50 or 75 min, taken in eleven separate observing sessions. The observations were made using a cryogenically cooled dual-channel system receiving orthogonal linear polarizations. For most of the post-1989 observations, the receiver was mounted in an off-axis location giving a system-equivalent flux density of ~95 Jy and a beamwidth of 35 arcmin. To allow removal of dispersion delays, the signals were passed through a 2×128 bank of 0.25-MHz filters and, after detection, low-pass filtering and summing of the two polarizations, they were sampled at 300- μ s intervals and recorded on magnetic tape.

A Convex C220 computer at the Australia Telescope National Facility, and an Alliant FX-8 and a Sun 330 Sparcstation at the Nuffield Radio Astronomy Laboratories were used for the off-line data analysis. The data were split into non-overlapping blocks of 2×10^6 and, separately, 8×10^6 samples (10.5 and 42 min duration respectively). After dispersion removal, the data were transformed using a fast Fourier transform routine. We searched for significant peaks in each spectrum and in the spectra obtained after summing 2, 4, 8 and 16 harmonics. This procedure was carried out for five separate dispersions in the range 23–27 cm^{-3} pc, around the measured value for PSR0021–72C. For promising suspects, the time-domain data were searched over a range of pulsar period and dispersion around the nominal values. An average pulse profile was then calculated for the optimum parameters.

PSR0021–72C was detected in about half of the observations. As found previously⁴, its intensity varies by more than a factor of ten from one observation to another, almost certainly a result of interstellar scintillations. A total of ten other pulsars were detected on two or more occasions. Typically, these pulsars were relatively strong for most of the observation when detected (signal-to-noise ratio of 15 or more), but were undetectable for most of the observations. These variations have a similar frequency and time structure to those observed for PSR0021–72C, and hence we attribute them to interstellar scintillation. All of the new pulsars are, on average, weaker than PSR0021–72C, and many of them would have been undetectable were it not for the scintillation.

Table 1 lists parameters for the new pulsars and for PSR0021–72C, included for completeness. Heliocentric periods and dispersion measures were obtained by taking a weighted mean of values from the time-domain analysis of observations where the pulsar detection was significant. Except for PSR0021–72C, the periods are uncertain in the last quoted digit, and uncertainties in the dispersion measures are typically $0.2 \text{ cm}^{-3} \text{ pc}$. Six of the new pulsars are definitely binary, and L may be binary. Within the errors, the other pulsars have constant heliocentric period, and we therefore believe them to be single, but it is possible that some of these also are members of long-period binary systems. Also listed in Table 1 are the numbers of observations in which the pulsar was significantly above the limiting sensitivity ($\sim 2.5 \text{ mJy}$ for an analysis of 2×10^6 points and a typical duty cycle) for at least part of the observation.

With this unprecedented number of millisecond pulsar detections, we were concerned that the pulsar signals were genuine and not due to some form of interference. The primary criteria for confirmation as a pulsar were that the signal-to-noise ratio should be high (>10) in at least one observation and, for single pulsars, that the heliocentric period should be the same in observations taken in different sessions. For binary pulsars, the heliocentric period and its rate of change were required to be consistent in different observations. Dispersion-measure determinations were, of course, also required to be consistent. As a further check that the signals were genuine, we analysed five 75-min observations taken immediately after observations of 47 Tuc, when the telescope was directed at PSR0540–69 in the Large Magellanic Cloud, using exactly the same analysis procedures and parameters as were used for the 47 Tuc analyses. None of the periodicities corresponding to pulsars listed in Table 1 was detected in these analyses. Finally, these periodicities have not been seen in numerous analyses of data for other globular clusters.

For PSR0021–72E and PSR0021–72J, the binary systems are of relatively short period, and the pulsars were detected on several occasions. This allowed unambiguous fitting of an orbital model to the period data, giving the results listed in Table 2. Uncertainties in the fitted quantities, given in parentheses, are

TABLE 1 Pulsars in 47 Tucanae

PSR	Pulse period (ms)	DM ($\text{cm}^{-3} \text{ pc}$)	Binary	No. of detections
0021–72C	5.756780	24.4	no	41
0021–72D	5.357573	24.7	no	14
0021–72E	3.536318	24.2	yes	12
0021–72F	2.623579	24.4	no	7
0021–72G	4.040379	24.2	no	2
0021–72H	3.2105	24.3	yes	6
0021–72I	3.48495*	23.7	yes	8
0021–72J	2.1006331	24.6	yes	10
0021–72K	1.7858	24.9	yes	6
0021–72L	4.34617	24.5	possibly	2
0021–72M	3.67666	24.4	yes	3

* Pulsar period may be twice this value.

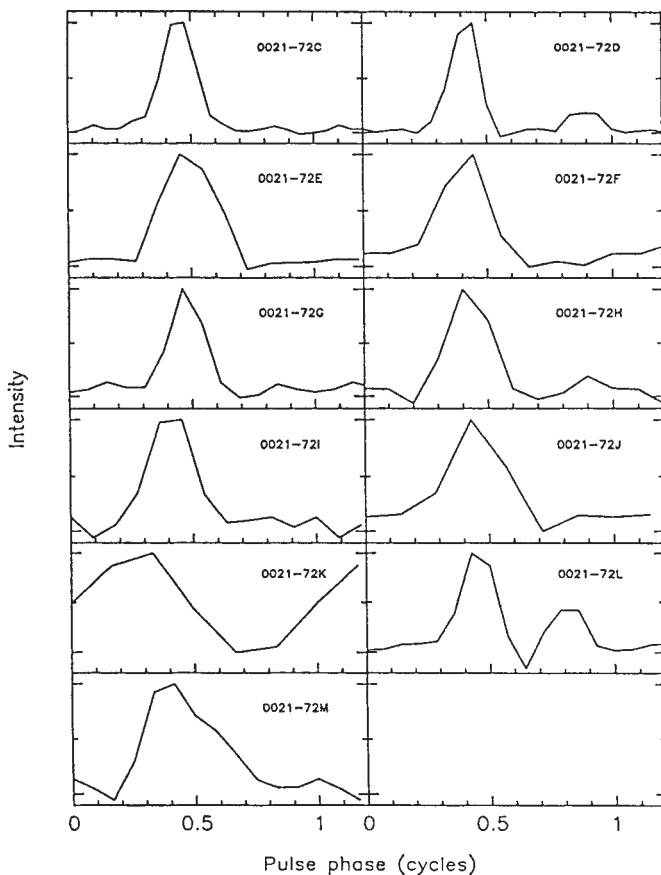


FIG. 1 Mean pulse profiles for the eleven pulsars detected in 47 Tuc. The profiles are scaled to have the same vertical extent.

in the last quoted digit. Although we have only limited data on PSR0021-72J so far, it has not been seen around orbital phase 0.25 when the pulsar is behind the companion. It is therefore likely that the pulsar is eclipsing and that, like PSR1957+20⁵ and PSR1744-24A⁶, PSR0021-72J is ablating its companion which has a mass of only $0.021 M_{\odot}$ (for an assumed pulsar mass of $1.4 M_{\odot}$ and orbital inclination of 90°). On the same assumptions, PSR0021-72E has a companion mass of $0.15 M_{\odot}$.

Pulse profiles for each of the pulsars listed in Table 1 are given in Fig. 1. Only three of the eleven pulsars (D, H and L) show evidence of an interpulse, although it is possible that the true period of I is twice the adopted value (Table 1) and hence that this pulsar has a main pulse and interpulse of comparable strength.

All of the new pulsars have dispersion measures close to that of PSR0021-72C. This demonstrates that PSR0021-72C and the new pulsars are located in 47 Tuc. It also makes it extremely unlikely that PSR0021-72A and B, reported by Ables *et al.*^{7,8}, which have the very different dispersion measure of $67 \text{ cm}^{-3} \text{ pc}$, are within the cluster (see also ref. 4). The observed range of dispersion measure seems similar to that for other globular clusters where more than one pulsar has been found (M5⁹, M15¹⁰⁻¹² and NGC6624¹³). Some or even all of these dispersion

differences may be due to differences along the various paths through the galactic interstellar medium. Hence we can only put an upper limit of $\sim 1 \text{ cm}^{-3}$ on the mean free electron density in the core region of the cluster.

A total of 19 pulsars were previously known in 12 different clusters, but of these only 13 are millisecond pulsars (arbitrarily defined to be those with periods of less than 25 ms). It is remarkable that all of the pulsars in 47 Tuc have periods of less than 6 ms. This contrasts strongly with M15, where three of the five pulsars¹⁰⁻¹² have periods of more than 30 ms, and NGC6624¹³ where one of the two pulsars has a period of 379 ms. Also, a higher proportion of the 47 Tuc pulsars are members of binary systems than in M5, M15 or NGC6624. As our survey is considerably more sensitive to single pulsars than to binaries, at least for orbital periods of less than a day, the true proportion of binary pulsars in 47 Tuc may even be larger than that indicated in Table 1. This high proportion of binaries and the absence of long-period pulsars may find a common solution related to their formation history. Their short periods place an upper limit of $\sim 2 \times 10^9$ years on their ages if they have magnetic field strengths of $10^{8.5}$ gauss, similar to those of other millisecond pulsars in globular clusters. Either these pulsars are the product of some event in the cluster that led to a burst of millisecond-pulsar formation about 10^9 years ago, or their luminosities fall rapidly with increasing period.

The high proportion of these pulsars in relatively short-period binary systems seems to favour the idea that millisecond pulsars acquire their rapid spin as a result of accretion in a low-mass X-ray binary system (LMXB)¹⁴. But the birthrate of such pulsars seems to exceed the birthrate of LMXBs by a large factor¹⁵, and this problem is exacerbated by the detection of such a large number of millisecond pulsars in this one cluster. 47 Tuc contains one X-ray source, which is believed to be a LMXB¹⁶. As LMXBs are strong X-ray sources, it is likely that this is the only LMXB in the cluster at present. The number of currently active pulsars in the cluster is difficult to determine, but it is almost

TABLE 2 Orbital parameters

	PSR0021-72E	PSR0021-72J
Heliocentric pulsar period (ms)	3.536318 (7)	2.1006331 (5)
Heliocentric orbital period (days)	2.22 (2)	0.120665 (2)
Semimajor axis (light-seconds)	2.00 (3)	0.0405 (6)
Eccentricity	<0.08	<0.03
Modified Julian day of ascending node	47861.17 (2)	48124.3546 (3)
Mass function (solar masses)	0.0017	4.9×10^{-6}

certainly many times the 11 detected so far. This suggests that the average active lifetime of a LMXB must be at least one and probably two orders of magnitude less than the average millisecond pulsar lifetime¹⁷⁻¹⁸. Alternatively, most of the pulsars may be formed by accretion-induced collapse of white dwarfs², or the current formation rate may be much less than that prevailing $\sim 10^9$ years ago.

Our results indicate that neutron stars may be a main component of the dark matter in this cluster, at least at the upper end of the range of 0.01–0.5% derived by Meylan³. They also imply that the initial mass function of the cluster was relatively flat and had an upper mass cutoff well above $10M_{\odot}$. We expect that the period derivatives of these pulsars will be affected by gravitational acceleration toward the core of the cluster (see also ref. 10), allowing an independent estimate of the mass distribution in the cluster. $\square$

Received 14 March; accepted 7 June 1991.

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ACKNOWLEDGEMENTS. We thank S. Johnston, X. Wu, G. Qiao, L. Staveley-Smith, B. Siegmund, D. Mar, K. Marvel and D. Berinson who at various times helped with the observations. N.D.A. acknowledges support from CNR-GIFCO and CNR Bilateral Projects. The Australia Telescope National Facility is operated in association with the Division of Radiophysics by CSIRO.

Evacuation of gas from globular clusters by winds from millisecond pulsars

D. N. Spergel

Princeton University Observatory, Princeton, New Jersey 08544, USA

WHY is so little gas observed within globular clusters? A typical globular cluster contains $\sim 10^3$ post-turnoff stars, each of which will lose $\sim 0.2M_{\odot}$ before its asymptotic giant branch phase of evolution, and $\sim 0.1M_{\odot}$ during this phase¹, and so should accumulate 10^2 – $10^3M_{\odot}$ of gas in the 10^8 -year interval between passages of the globular cluster through the galactic disk. (At each disk passage, gas ram pressure will remove the accumulated material^{2,3}.) But observational searches show that there is scant intracluster gas; in many clusters, there is less than $1M_{\odot}$ of gas, orders of magnitude less than theoretical predictions³. The recent discovery^{4,5} of multiple millisecond pulsars in globular clusters may resolve this long-standing problem: the relativistic wind from these pulsars is enough to drive gas from stellar mass loss out of the globular cluster.

Despite considerable observational effort, very little gas has been detected in globular clusters. Searches for neutral hydrogen have failed to detect any gas in globular clusters^{6–10}. Attempts to detect ionized gas through its free-free emission have produced only null results^{11–13}. Millimetre-wavelength searches have not found any intracluster molecular gas^{14–15}. There was no dust found by the infrared astronomy satellite IRAS in any

of the galactic globular clusters¹⁶. In many clusters, there seems to be less than $1M_{\odot}$ of gas².

This discrepancy between the low upper limits on gas content and the theoretical expectations of substantial gas content has prompted a series of theoretical proposals for removal mechanisms that can operate between passages of the clusters through the galactic disk. These mechanisms range from rapid stellar winds¹⁷ or nova-driven winds¹⁸ to continuous sweeping of the clusters by a hot galactic halo^{19–21}. There is, however, no convincing evidence for any of these mechanisms³.

The recent detection of multiple millisecond pulsars¹ in globular clusters suggests a resolution of this dilemma. A recent study of 47 Tuc by Lyne, Manchester and collaborators⁵ led to the reported detection of 10 pulsars with millisecond periods. Kulkarni, Narayan and Romani²² estimate that there are several 'recycled pulsars' in a typical globular cluster with magnetic fields of $B \approx 10^9$ – 10^{10} gauss. The luminosity of a single 3-ms pulsar with a 10^9 -gauss field is $\sim 3 \times 10^{35}$ erg/s⁻¹. (A pulsar radiating at this rate will spin down in the age of the universe.) If only a small fraction of $\sim 10^{36}$ – 10^{37} erg s⁻¹ radiated by the dozens of millisecond pulsars in a globular cluster is transferred to the intracluster medium, then the pulsars can easily provide the 10^{34} erg s⁻¹ needed to remove the $10^{-5}M_{\odot}$ per year injected into the cluster by stellar mass loss into the cluster potential well of ~ 40 km s⁻¹ (ref. 2).

Will the energy emitted by the pulsar interact with the intracluster gas? Pulsars are observed to emit a fraction, f , of their energy in X-rays and γ -rays. Pulsar models predict that the remaining luminosity is emitted either in a relativistic wind or in low-frequency radiation. In the Crab nebula, this wind is observed to be accelerating the surrounding gas shell²³. Models of rapidly rotating pulsars²⁴ suggest that only a small fraction, $f \ll 1$, of the pulsar radiation is in X-rays and γ -rays.

In a globular cluster, where most of the pulsars are concentrated in the core, this wind will have a ram pressure of $L_{\text{pulsar}}(1-f)/4\pi R^2 c \approx 10^{-12}$ dynes at 1 pc from the cluster. Whether the wind consists of relativistic particles or low-frequency radiation, it will interact with any dense gas in the cluster. The ram pressure will balance the expansion pressure of mass loss from a red giant star losing $10^{-7}M_{\odot}$ per year at 10 km s⁻¹ at a radius of 0.2 pc. The interaction of the two winds will produce a cometary-like structure with the gas flowing away from the star. This interaction may be similar to the interaction between the wind from PSR1957+20 and the interstellar medium inferred from observations of H α from the surrounding nebula²⁵.

Radio observations of the planetary nebula K648 in M15 may show direct evidence for the interaction between the pulsar wind and the planetary nebula gas. The nebula appears asymmetric²⁶ with a tail pointing away from the pulsars^{27,28} in the cluster centre. More detailed studies of K648 may provide a powerful test of this cluster cleaning mechanism. $\square$

Received 15 April; accepted 5 June 1991.

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ACKNOWLEDGEMENTS. I thank J. Applegate, C. Bailyn, B. Draine, G. Knapp, J. Ostriker, R. Romani, and S. Thorsett for helpful discussions. I thank the Sloan Foundation and the NSF for support.

Superconductivity at 33 K in $\text{Cs}_x\text{Rb}_y\text{C}_{60}$

K. Tanigaki, T. W. Ebbesen, S. Saito, J. Mizuki, J. S. Tsai, Y. Kubo & S. Kuroshima,

Fundamental Research Laboratories, NEC Corporation, Tsukuba 305, Japan

THE synthesis of macroscopic quantities^{1,2} of the fullerenes C_{60} and C_{70} has led to discoveries of several unusual properties^{3–10}, in particular the high conductivity³ and superconductivity^{4–6} of alkali-metal-doped phases. Here we report a superconducting phase of C_{60} doped with caesium and rubidium, which has the highest transition temperature T_c and the largest diamagnetic shielding found so far for the alkali-metal-doped compounds. $\text{Cs}_x\text{Rb}_y\text{C}_{60}$ ($x=2$ and $y=1$ in the dopant feed) exhibits a T_c of 33 K and a diamagnetic shielding of over 60%. This is also the highest T_c yet observed in a molecular superconductor. The variation of T_c with dopant, $T_c(\text{K}_x\text{C}_{60}) [18 \text{ K}] < T_c(\text{Rb}_x\text{C}_{60}) [29 \text{ K}] < T_c(\text{Cs}_x\text{Rb}_y\text{C}_{60}) [33 \text{ K}]$, supports the interpretation that the transition temperature of these fullerenes is determined mainly by the density of states at the Fermi level.

Hebard *et al.*⁴ first reported evidence of superconductivity in potassium-doped C_{60} , which has a transition temperature of 18 K. Subsequently, Rosseinsky *et al.*⁶ and Holczer *et al.*⁵ reported independently that T_c increases to 28 K and 30 K, respectively, when rubidium is the dopant. Caesium-doped C_{60} was studied with the Rb-doped material, but superconducting properties were not observed⁵. We reasoned that a larger alkali metal should produce a larger lattice constant, which should in turn increase the density of states at the Fermi level and thus yield a higher T_c . The size of interstitial sites in the C_{60} crystal, however, is such that only one, or perhaps two, Cs atoms can be accommodated without disrupting the close-packing. We have therefore explored the possibility of doping with various proportions of Rb and Cs.

C_{60} was purified chromatographically using a neutral alumina column (activity I) with toluene/hexane elutants, after ether treatment of the arc-generated carbon soot produced according to the method of ref. 2. The purity of the C_{60} was confirmed to be at least 99.9% by infrared, ultraviolet and mass spectroscopies. The C_{60} (9 mg) was placed in a pyrex tube (5 mm diameter) together with Rb and/or Cs. The small amounts of alkali metal were measured in metal-filled glass capillary tubes (0.5 mm diameter), which were cut and handled under a helium atmos-

TABLE 1 T_c and fractional shielding for various nominal dopant ratios

Dopant	T_c (K)	Fractional shielding [†]	
		ZFC	FC
K_3C_{60}	18	47%*	–
Rb_2C_{60}	29	14%	6%
Rb_3C_{60}	29	10%	4%
Rb_4C_{60}	29	18%	5%
$\text{Cs}_1\text{Rb}_2\text{C}_{60}$	31	30%	13%
$\text{Cs}_2\text{Rb}_1\text{C}_{60}$	33	60%	15%

* Ref. 5.

† ZFC: zero-field cooled; FC: field cooled.

phere in a glove box. The pyrex tubes containing C_{60} and the dopant were degassed to 10^{-2} torr and refilled with helium (700 torr). The degassing/refilling process was repeated twice before sealing the tubes. These were then heated at 390 °C for 74 h. The temperature dependence of the d.c. magnetization of the samples was measured with a SQUID magnetometer (Quantum Design MPMS).

After cooling $\text{Cs}_x\text{Rb}_y\text{C}_{60}$ ($x=2$, $y=1$ in the feed) to 4 K in zero field, a magnetic field of 10 Oe was applied and the magnetic susceptibility χ was measured while warming to ~ 37 K. On warming, the applied field is excluded by the sample up to 33 K (Fig. 1). The existence of the superconducting phase was then confirmed unambiguously by measuring the Meissner effect on field-cooling. The onset of a well defined Meissner effect was observed at 33 K (Fig. 1). The same measurements were performed on $\text{Cs}_x\text{Rb}_y\text{C}_{60}$ ($x=1$, $y=2$ and $x=0$, $y=3$ in the feed), for which a Meissner effect was observed with a T_c of 31 K and 29 K respectively (Table 1). The latter value is consistent with the published values of 28 K (ref. 6) and 30 K (ref. 5) for rubidium-doped C_{60} . The negative sign of χ at $T < T_c$ for zero-field cooling, and the Meissner effect on field cooling, can be explained only by the appearance of a superconducting phase. From the magnitude of the zero-field-cooled curve, the fractional diamagnetic shielding of the superconductor is above 60% (lower estimate) for $\text{Cs}_x\text{Rb}_y\text{C}_{60}$ (with nominal ratio $x=2$ and $y=1$). All of our samples showed shieldings between 10% and 60% of that expected for a homogeneous superconductor. This is much larger than the values reported for Rb_xC_{60} by other groups (1%⁶, 7%⁵). The only comparable shielding value reported previously is for K_xC_{60} (47%⁵). For Cs_xC_{60} (with $x=3$ in the feed), no superconducting transition was observed, in agreement with earlier observations⁵.

Zero-field-cooled data for five superconducting samples of $\text{Cs}_x\text{Rb}_y\text{C}_{60}$ with varying x and y are shown in Fig. 2. When y in the feed was varied between 2 and 4 in the absence of Cs, the onset T_c remained at 29 K but the relationship between y and the diamagnetic fraction was not simple. This contrasts with K-doped C_{60} , for which the maximum superconducting fraction is observed at the nominal composition K_3C_{60} (ref. 5). The composition of the superconducting Rb-doped phase is probably also Rb_3C_{60} , but as the reactivity and diffusivity of Rb are less

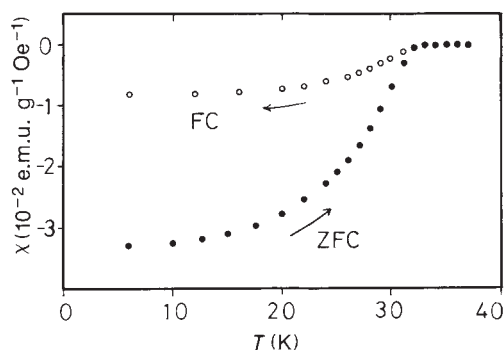


FIG. 1 Magnetic susceptibility χ as a function of temperature for the sample with nominal composition $\text{Cs}_2\text{Rb}_1\text{C}_{60}$.

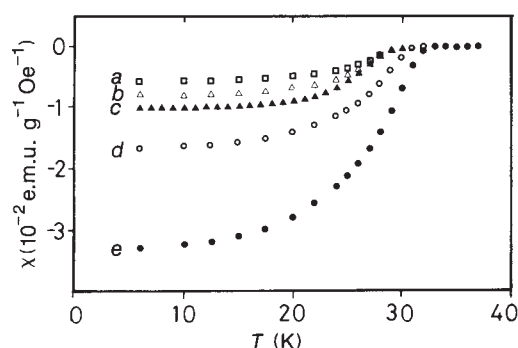


FIG. 2 Zero-field-cooled magnetic susceptibility $\chi(T)$ for various nominal compositions of $\text{Cs}_x\text{Rb}_y\text{C}_{60}$ (a: $x=0$, $y=3$; b: $x=0$, $y=2$; c: $x=0$, $y=4$; d: $x=1$, $y=2$; e: $x=2$, $y=1$).

than K, a homogeneous distribution of the alkali metal throughout the sample is probably more difficult to achieve in the latter case. It could therefore be difficult to establish a correlation between the initial stoichiometric proportions in the feed and those in the superconducting fraction. Although the curves in Fig. 2 exhibit broad transitions at the onset of superconductivity (like those of typical copper oxide superconductors), there are no obvious indications of two or more distinct transitions. Furthermore, as Cs doping alone does not produce a superconducting phase, the increase in T_c observed on gradually increasing the Cs/Rb ratio must be due to a superconducting phase that contains both Cs and Rb.

In a face-centred-cubic C_{60} lattice, the unit cell contains two tetrahedral and one octahedral interstitial site. These can accommodate spherical ions of ~ 1.1 Å and 2.1 Å respectively. It therefore seems unlikely that the lattice can accommodate three Cs ions (radius 1.7 Å) per unit cell without significantly disrupting the structure. This is the most likely explanation for why we do not observe superconductivity in Cs_xC_{60} . Alternatively, it is possible that Cs cannot diffuse or penetrate into the C_{60} lattice because of its large size. This argument is weakened, however, by the evidence here that mixed Cs/Rb doping increases T_c and yields a very large diamagnetic shielding of the superconducting phase.

A recent study¹⁰ on K_3C_{60} has revealed that the Fermi level is located close to a peak in the density of states, suggesting that a conventional BCS-type mechanism might operate in alkali-metal-doped fullerenes. The increase in T_c with increasing alkali-metal ionic radius may be explained by the expected concomitant increase in the lattice constant, thereby reducing the dispersion at the Fermi level owing to interactions between neighbouring C_{60} anions: this in turn will increase the density of states at the Fermi level, resulting in a higher T_c . This idea is further supported by the fact that the Raman frequencies for doped M_xC_{60} (where M is Na, K, Rb or Cs) films are very similar⁶, indicating little change in intramolecular phonon modes. We are now attempting to measure lattice constants to verify this idea.

The high T_c (exceeded only by copper oxide superconductors) and large volume fraction of the $\text{Cs}_x\text{Rb}_y\text{C}_{60}$ phase suggests that further improvements may be expected. In particular, on both theoretical and experimental grounds, mixed dopants seem very promising for optimizing these properties. □

Superconductivity at 30 K in caesium-doped C_{60}

Stephen P. Kelty, Chia-Chun Chen
& Charles M. Lieber*

Department of Chemistry and Division of Applied Sciences,
Harvard University, Cambridge, Massachusetts 02138, USA

RECENTLY there has been significant effort directed towards exploring the physical and chemical properties of C_{60} and other large carbon clusters^{1–11}. Particularly intriguing are reports of superconductivity in potassium- and rubidium-doped C_{60} crystals and films^{5–7}. The transition temperatures (T_c) for K-doped C_{60} (18 K) and Rb-doped C_{60} (~ 28 K) are significantly higher than those reported previously for other molecular superconductors¹². Earlier attempts to prepare a caesium-doped superconducting phase⁷ have proved unsuccessful. Here we report that a Cs-doped superconductor can be prepared by using as the dopant binary alloys of the type CsM (where M is Hg, Tl or Bi). We observe a reproducible superconducting transition in Cs_xC_{60} ($x=1.2–3$) at 30 K, demonstrated by flux expulsion (the Meissner effect) and flux exclusion (shielding) d.c. magnetization measurements. The low superconducting volume fraction ($\sim 1\%$) suggests that further studies will be needed to determine the optimal doping concentration and to place tighter bounds on T_c .

Reported syntheses of K- and Rb-doped C_{60} superconductors have relied on the direct reaction of alkali-metal vapour with C_{60} . This approach has resulted in large superconducting fractions for the K-doped materials⁷, but much smaller fractions in the Rb-doped system^{6,7}. Successful preparation of superconducting Cs-doped C_{60} has not yet been reported, although recent structural studies¹³ have shown that it is possible to prepare a Cs_6C_{60} compound by direct reaction of Cs vapour and C_{60} . Unfortunately, the stoichiometry of this latter material is significantly greater than that expected for the superconducting phase (approximately M_3C_{60})^{5,7} and the corresponding structure is distinct from that of the superconducting phase determined¹⁴ for K_3C_{60} . To enable greater control of the Cs doping process, we took a new approach which uses binary alloys of caesium, CsM (where M is Hg, Tl or Bi), which are less reactive than pure caesium metal.

C_{60} was synthesized in a stainless-steel chamber using high-purity graphite electrodes (99.9995%), as described in refs 1 and 2. The carbon soot produced from an arc discharge in 100–150 torr of helium was extracted with benzene, and pure C_{60} was obtained from this solution by chromatography on neutral alumina¹⁵. The chromatography and subsequent isolation of C_{60} was carried out in the dark to minimize impurities resulting from the photodegradation of C_{60} . The C_{60} was then dried under vacuum for several hours (200–250 °C) to remove solvent. The CsM_x (M = Hg, Tl, Bi) and the $(\text{CsM}_x)_y\text{C}_{60}$ doped materials were prepared in an inert-atmosphere glove box equipped with an $\text{O}_2/\text{H}_2\text{O}$ removal system, and then sealed on a vacuum line without exposing the samples to air.

Temperature-dependent magnetic susceptibility measurements obtained from a $(\text{CsTl}_2):\text{C}_{60}$ (ratio 1.2:1) sample using a SQUID magnetometer are shown in Fig. 1. The sample was prepared from 10.3 mg CsTl_2 and 11.4 mg C_{60} , and was reacted at 220 °C. After 1 h of reaction, the zero-field-cooled curve obtained on warming the sample from 5 K in a field of 50 Oe exhibits a clear transition at 29.5 K: above 29.5 K, magnetic flux is no longer excluded from the sample. On cooling the sample from 50 K in a 50-Oe field, we observe flux expulsion from the sample (Meissner effect) with an onset at 29.5 K. The field-cooled and zero-field-cooled curves are completely stable to

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ACKNOWLEDGEMENTS. We thank A. Oshiyama and N. Hamada for discussions and Y. Shimakawa for assistance with the SQUID measurements.

* To whom correspondence should be addressed.

repeated cycling of the sample between 5 and 300 K. These flux expulsion and exclusion data are not consistent with any phenomena other than superconductivity, and thus we assign to the transition a T_c of 29.5 K.

Previous studies of graphite intercalation complexes made from alkali-metal/M (M = Hg, Tl, Bi) alloys have shown that these reactions lead to ternary intercalation complexes¹⁶. The formation of ternary C_{60} compounds ($CsMC_{60}$) would be interesting in itself, but our results show that such compounds do not form under the present reaction conditions. Specifically, we find that reactions of C_{60} with $CsHg_{1.1}$ and $CsBi$ alloys in a $CsM_x:C_{60}$ ratio of 3:1 leads reproducibly to the formation of superconducting compounds with transition temperatures of 29 ± 1 K (Fig. 2). As nearly the same T_c is observed from C_{60} reactions with $CsHg_{1.1}$, $CsTl_2$ and $CsBi$, we believe that the only reasonable conclusion is that an identical superconducting phase is formed in all three reactions, which must correspond to Cs_xC_{60} . In further support of this assignment, we note that reactions of KM and RbM alloys with C_{60} produce the known K_3C_{60} and Rb_3C_{60} superconducting phases (S.P.K., C.C.C and C.M.L., unpublished results).

We can also rule out the possibility that the observed T_c is due to rubidium impurity on several grounds. First, trace analysis of the starting materials (by inductively coupled plasma emission spectroscopy) shows that rubidium is present at ≤ 10 p.p.m., much below the quantity that would be required to account for the observed superconducting fraction. Second, we have obtained similar results from three independent sources of Cs and three different alloys ($CsHg$, $CsTl_2$ and $CsBi$) prepared from these caesium sources. Finally, our magnetic measurements on independently prepared Rb_3C_{60} samples show that the T_c of the Rb-doped material (27–28 K) is 1–2 K lower than that observed for Cs-doped C_{60} .

We have investigated the stability and composition of the Cs_xC_{60} superconducting phase. Measurements of the shielding signal as a function of reaction time indicate that 1–3 h of reaction is optimal for the $CsHg_{1.1}$ (300 °C), $CsTl_2$ (200 °C) and $CsBi$ (200 °C) alloys; longer reaction leads to a systematic decrease in the superconducting fraction. These observations indicate that the superconducting phase is less stable than the K- and Rb-doped superconductors, because these latter materials can be heated for at least two days without degradation of the superconducting phase. The instability of the Cs-doped C_{60} superconducting phase is not unexpected, however, in view

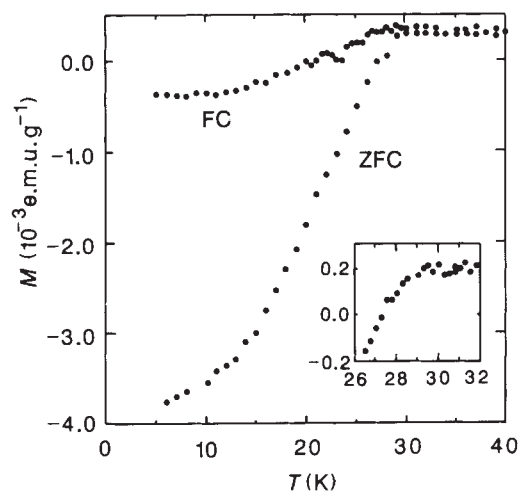


FIG. 1 Temperature dependence of the magnetization obtained for a $(CsTl_2)_{1.5}C_{60}$ sample after 1 h of reaction. The curves were obtained by cooling in zero field and subsequent warming in a 50-Oe field (ZFC) and by cooling in a 50-Oe field (FC). The inset shows clearly the T_c of 29.5 K.

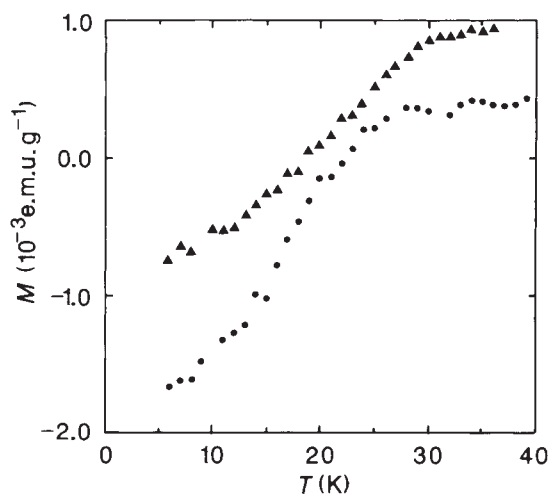


FIG. 2 Zero-field-cooled magnetization as a function of temperature for a $(CsHg_{1.1})_3C_{60}$ sample after 1 h of reaction at 300 °C ($\blacktriangle$), and a $(CsBi)_3C_{60}$ sample after 1 h of reaction at 200 °C ($\bullet$). The curves were determined in a 50-Oe field.

of the fact that direct reaction of Cs with C_{60} fails to yield superconducting material. We believe that formation of the Cs_xC_{60} superconducting phase is kinetically favoured in CsM alloy reactions, but that this phase undergoes a structural transformation to yield domains of the thermodynamically stable but non-superconducting Cs_6C_{60} compound that has been recently characterized¹³.

Studies of the composition dependence of the Cs_xC_{60} superconducting phase provide further insight into the stability and stoichiometry, x , of this system. We find for $CsTl_2:C_{60}$ ratios greater than 3:1 that the superconducting fraction decreases rapidly. No superconducting material is detected at a 6:1 stoichiometry. For $CsTl_2:C_{60}$ and $CsBi:C_{60}$ ratios between 1.2:1 and 3:1 we obtain similar yields ($\sim 1\%$) of the superconducting phase, although the largest superconducting fractions have been obtained at the 3:1 stoichiometry. Our present data indicate that the optimal stoichiometry is between 1.2 and 3:1 ($Cs:C_{60}$), although by analogy with K_3C_{60} we speculate that 3:1 is the stoichiometry of the superconducting phase. The low yield of superconducting phase is believed to be due to the transformation of this phase, under our present reaction conditions, to a non-superconducting one, as discussed above. Future investigations of the dependence of the superconducting fraction on temperature, alloy composition and reaction time will undoubtedly lead to higher yields.

Within the context of the BCS theory of superconductivity, T_c will depend exponentially on the density of states at the Fermi level, $N(E_F)$, and the effective electron coupling, V_0 . If we assume that the K, Rb and Cs superconducting phases are isostructural, then doping of larger ions into the lattice should reduce V_0 , but at the same time increase $N(E_F)$ because of the decreased interaction between C_{60} clusters and consequent narrowing of the conduction band. One can speculate that an increase in $N(E_F)$ overcomes any decreases in V_0 on going from K to Rb (ref. 6), but that for Cs V_0 decreases sufficiently to offset the expected increases in $N(E_F)$. In view of the low superconducting volume fraction reported here and the uncertainty in exact stoichiometry of the superconducting phase, our value of $T_c \approx 30$ K may be a lower bound for Cs-doped C_{60} . □

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Partial oxidation of methane to synthesis gas using carbon dioxide

A. T. Ashcroft*, A. K. Cheetham*, M. L. H. Green† & P. D. F. Vernon†

* Chemical Crystallography Laboratory, University of Oxford, 9 Parks Road, Oxford OX1 3PD, UK

† Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, UK

INCREASING concern about world dependence on petroleum oil has generated interest in the more efficient use of natural gas^{1–4}. The conversion of methane to the common feedstock synthesis gas (carbon monoxide and hydrogen) by steam reforming is already well established⁵, and we have shown recently that yields of synthesis gas in excess of 90% can be obtained at moderate temperatures and ambient pressure by partial oxidation, with air or oxygen, over supported transition-metal catalysts^{6,7}. The use of carbon dioxide as an oxidant for conversion of natural gas to synthesis gas is well established in steam reforming⁵, and is also known in CO₂ reforming (for example, the Calcor process^{8,9}), in which the use of excess CO₂ yields mainly CO. In the present work, we describe an alternative catalytic strategy for CO₂ reforming which gives excellent yields (90%) from a stoichiometric (1:1) feed of CO₂ and CH₄. Carbon deposition ('coking'), which is a hazard of CO₂-reforming routes, is suppressed here by the use of catalysts based on platinum-group metals. We show that the exothermic partial oxidation of CH₄ and the endothermic CO₂-reforming reaction can be carried out simultaneously, thus introducing the possibility of tuning the thermodynamics of the process.

The principal source of synthesis gas, which is used as a feedstock for methanol synthesis and Fischer–Tropsch conver-

TABLE 2 Results of catalytic reactions with CH₄ and CO₂ mixtures of different compositions over 1% Ir/Al₂O₃ at 1,050 K

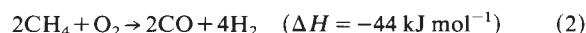
CO ₂ /CH ₄	Methane converted (%)	CO ₂ converted (%)	Yield H ₂ (%)	Yield CO (%)
3.84	100	41	70	54
2.96	99	50	76	62
1.99	97	62	83	74
1.00	88	91	87	89
0.60	58	96	57	71
0.49	47	98	47	64
0.35	34	100	34	51

Total gas hourly space velocity, $2 \times 10^4 \text{ h}^{-1}$; pressure 1 atm. Yields of H₂ and CO defined as in Table 1.

sions, is the steam-reforming reaction



but the partial oxidation reaction



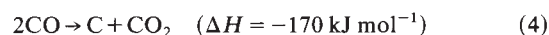
has several advantages over steam reforming, notably its greater selectivity to synthesis gas production, its exothermicity, and the more desirable CO/H₂ ratio of the product. The corresponding carbon dioxide reforming reaction



has been previously studied^{10,11} and is widely used in secondary reforming processes to reduce the H₂/CO ratio obtained by steam reforming⁵. The CO₂/CH₄ reaction, which is highly endothermic and prone to carbon deposition, has recently been commercialized by using a CO₂-rich feedstock over an unspecified catalyst^{8,9}. It is also being examined for chemical heatpipe technology^{12–15}, in which a power source (for example, solar or nuclear) drives an endothermic reaction such as (3) above, and the product gases are transported to the consumer, where the exothermic reverse reaction, in this case 'methanation', is initiated.

We initially examined the stoichiometric CO₂-reforming reaction, equation (3), at 1,050 K and atmospheric pressure over a number of supported transition metals (Table 1) using a catalytic testing procedure similar to that employed previously^{6,7}. Clearly, the reaction proceeds well over a number of catalysts, producing synthesis gas yields of almost 90%, which are significantly better than previously reported values^{11,14}.

Under these conditions of stoichiometric CO₂ reforming, thermodynamic considerations predict carbon deposition by the Boudouard reaction^{16–18}:



In our own and previous studies¹⁹, it has proved impossible to avoid carbon formation under such low CO₂/CH₄ ratios using nickel catalysts. Sulphur passivation, blocking some of the active sites to inhibit carbon formation, has been commercialized in steam reforming as the SPARG process²⁰. The passivation process works to a certain extent²¹, but at the expense of catalytic activity. Here we use highly dispersed noble metal catalysts, prepared by a vacuum 'incipient wetness' technique described previously⁷, which retain sufficient activity to reform the methane and carbon dioxide efficiently, while suppressing carbon deposition. The thermodynamic driving force towards carbon is so strong that even palladium, generally a good catalyst for carbon-free reforming²², cokes after only a few hours (Table 1). Only iridium and rhodium retain their activity for 200 hours in a silica reactor, the used catalysts containing less than 0.3% carbon.

The catalytic performance of the Ir/Al₂O₃ catalyst under a range of feedstock ratios is illustrated in Table 2, which shows that either of the components may be entirely converted in the

TABLE 1 Results of catalytic reactions at 1,050 K

Catalyst	Methane converted (%)	CO ₂ converted (%)	Yield† of H ₂ (%)	Yield‡ of CO (%)	Coking rate (gC per g Cat per h)
Al ₂ O ₃	1	2	0	1	0
Ni/Al ₂ O ₃ §	88	81	88	85	0.8
1% Pd/Al ₂ O ₃	71	75	69	73	0.1
1% Ru/Al ₂ O ₃	67	71	62	69	0
1% Rh/Al ₂ O ₃	86	88	85	87	0
1% Ir/Al ₂ O ₃	88	91	87	89	0

Reactant gas flow rates: CH₄, 5 ml min⁻¹; CO₂, 5 ml min⁻¹. Total gas hourly space velocity, $1.7 \times 10^4 \text{ h}^{-1}$; pressure 1 atm.

* Catalyst samples (50 mg) were prepared by incipient wetness techniques from the transition-metal chlorides. This and the catalytic testing procedure were similar to that described previously^{6,7}.

† Yield H₂ = (amount of H₂ in products)/(2 × amount of CH₄ in reactants)

‡ Yield CO = (amount of CO in products)/(amount of (CO₂ + CH₄) in reactants)

§ CRG 'F' steam-reforming catalyst, from British Gas.

TABLE 3 Results of catalytic reactions of a CH₄/CO₂ mixture (1:1 ratio) over 1% Rh/Al₂O₃ at 1,050 K

GHSV 10 ⁴ h ⁻¹	Methane converted (%)	CO ₂ converted (%)	Yield H ₂ (%)	Yield CO (%)
0.5	88	91	87	89
1.0	88	91	87	89
1.7	86	88	85	87
2.4	85	87	83	86
5.6	68	74	68	71

Total pressure 1 atm. Yields of H₂ and CO defined as in Table 1. GHSV, gas hourly space velocity.

presence of a large excess of the other, with no carbon deposited under any of the conditions examined. With a stoichiometric (1:1) feed, nearly 90% of the CO₂ and CH₄ are converted to synthesis gas, a finding that may have important environmental implications, as two greenhouse gases are converted into a valuable feedstock. Together with the partial oxidation and steam-reforming reactions, the mechanism is assumed to be based on the equilibration of the CO₂-reforming reaction (3), the steam-reforming reaction (1) and the reverse water-gas shift reaction below.



Calculations of these thermodynamic equilibria predict a slightly greater (93%) conversion of carbon dioxide, and a slightly lower (97%) selectivity to hydrogen than was observed experimentally, suggesting that the water-gas shift equilibrium is not quite achieved. As expected, synthesis gas formation becomes more efficient as the temperature is increased, yields improving from <10% at 800 K to 88% at 1,050 K. Table 3 shows that increasing the flow rate over 1%Rh/Al₂O₃ produces a decrease in the catalytic conversion. But as it is the temperature of the reactor, and not the catalyst itself, that is measured, it is unclear whether this is due to the reduction in residence time, or the effect on the catalyst temperature of passing high flows of relatively cool gases (input temperature 400 K) for what is already an endothermic reaction.

Noting that both the partial oxidation and CO₂ reforming reactions will proceed with high yields over supported iridium, we then examined the combined reaction with a variety of feedstocks. The results of these extremely successful experiments are detailed in Table 4, showing synthesis gas yields of up to 90%. In this manner, therefore, the exothermic partial oxidation reaction and the endothermic CO₂-reforming reaction can be combined to achieve a thermally neutral reaction which may be advantageous from an engineering viewpoint. This may also make it easier to use natural gas reserves that are contaminated with substantial quantities of CO₂. Again, nickel-containing catalysts deposit carbon extremely rapidly, except when excess CO₂ is used: for example, with a feed of CH₄:O₂:CO₂ in a ratio of 4:1:9, at near total methane conversion, the exit gas composition approaches 50% CO at 1,050 K and no carbon is observed. □

TABLE 4 Results of catalytic reactions with mixtures of CH₄, O₂ and CO₂ of different compositions over 1% Ir/Al₂O₃ at 1,050 K

Feed composition (%)			CH ₄ converted (%)	CO ₂ converted (%)	Yield† H ₂ (%)	Yield CO (%)
CH ₄	CO ₂	O ₂				
64.4	3.5	32.1	92	9	89	86
59.4	20.0	20.6	87	83	81	86
58.3	23.7	18.0	84	83	81	84
58.0	28.0	14.0	83	90	79	85
49.8	48.8	1.4	91	87	91	89

Total gas hourly space velocity 2 × 10⁴ h⁻¹; pressure 1 atm. Yields of H₂ and CO defined as in Table 1. In all cases, oxygen conversions were >99.7%.

Received 11 February; accepted 29 May 1991.

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ACKNOWLEDGEMENTS. We thank Johnson Matthey for the loan of the precious metal chlorides. A.T.A. thanks British Gas and the SERC for a studentship and P.D.F.V. thanks BP for a studentship.

Use of cosmogenic ³⁵S to determine the rates of removal of atmospheric SO₂

N. Tanaka & K. K. Turekian

Department of Geology and Geophysics, Yale University, PO Box 6666, New Haven, Connecticut 06511, USA

GASEOUS sulphur dioxide supplied to the atmosphere is removed principally by three processes: direct scavenging in precipitation, oxidation to aerosol sulphate with subsequent deposition by vertical and horizontal precipitation, and 'dry' deposition, primarily on the surface of vegetation. The rates of these removal processes, which vary with environmental conditions, must be known in order to understand the fate of SO₂ and the concentration and distribution of aerosol sulphate^{1–3}. The latter is thought to play a part in the heat balance of the lower troposphere⁴, and is thus relevant to the issue of global warming. Approaches to this problem using field observations^{5,6} have not given consistent or uncontested results. We report here the use of cosmogenic ³⁵S (half-life 87.2 days) as a way of determining the time constants for oxidation, in-cloud scavenging and aerosol deposition. Our method involves determining ³⁵S levels in gaseous SO₂, aerosol sulphate and precipitation. If these seasonally and regionally variable time constants can be applied to terrestrially produced SO₂, ³⁵S measurements could provide an independent method for studying the fate of SO₂ in the atmosphere as a function of time and place.

Radioactive ³⁵S (β-decay to ³⁵Cl) is produced in the atmosphere by the bombardment of ⁴⁰Ar with cosmic rays and has been observed in wet precipitation^{7–9}. Junkermann and Roedel¹⁰ argue that most ³⁸S (and by analogy ³⁵S) produced by cosmic rays is rapidly converted to ³⁸SO₂, basing this argument on their evaluation of the kinetics of likely reactions, although Calvert *et al.*¹¹ think that, at least for ³⁸S (half-life 2.84 hours) produced at ground level, direct formation of the sulphate may occur. The effective tropospheric production of ³⁵S at any location is the sum of the actual *in situ* tropospheric production and the fraction of the stratospheric reservoir of ³⁵S that is injected into the troposphere. This fraction is geographically and seasonally variable, as inferred from the study of nuclear-bomb-injected radionuclides over the past 30 years.

Our measurements are made at the Earth's surface and are therefore in the planetary boundary layer, whose vertical extent

varies with local climate. If we treat the boundary layer in a broad region as a simple box, the ^{35}S concentrations in gaseous and particulate phases are governed by two equations. The equation for $^{35}\text{SO}_2$ is

$$dS_0/dt = P - \lambda S_0 - jS_0 - cS_0 - vS_0 \quad (1)$$

where S_0 is the concentration of $^{35}\text{SO}_2$ in air, P is the effective tropospheric production rate, and λ is the radioactive decay constant of ^{35}S ; j , c and v are the first-order rate constants for the removal of SO_2 from the air by conversion to sulphate, precipitation scavenging and 'dry' deposition, respectively.

The ^{35}S in aerosol sulphate is regulated by the following equation (assuming that the removal process is first order)

$$dS_p/dt = jS_0 - \lambda S_p - kS_p \quad (2)$$

where S_p is the $^{35}\text{SO}_4^{2-}$ concentration in air as aerosol, and k is the scavenging constant for aerosol removal, primarily by precipitation.

If we assume steady state, the equations reduce to two simultaneous equations with seven unknowns. If we measure S_0 and S_p , the number of unknowns reduces to five. The P term can be partitioned into that fraction collected in a bucket and that collected as 'dry' deposition, presumably mainly on the surfaces of vegetation:

$$P \approx P_{j+c} + P_v \quad (3)$$

and

$$P_v = vS_0 \quad (4)$$

As P_{j+c} can be measured in buckets, and $P_v = vS_0$, then equation (1) becomes (assuming steady state):

$$0 = dS_0/dt = P_{j+c} - \lambda S_0 - jS_0 - cS_0 \quad (5)$$

This reduces the unknowns in equations (1) and (5) to j , c and k . If we have one more equation relating these unknowns, their values are uniquely determined.

The relationship between c and k can be independently determined. W. C. Graustein and K. K. T. (unpublished data, available from the authors) have determined the average rate of in-cloud and raindrop scavenging of SO_2 , relative to precipitation of aerosol SO_4^{2-} , by using ^{210}Pb and sulphate measurements in aerosols and precipitation (^{210}Pb is produced from ^{222}Rn in the atmosphere and exists there only in submicrometre aerosols). The flux of aerosol sulphate can be determined by measuring the ^{210}Pb flux to a bucket over time (or using a soil profile as a time integrator) and the ratio of sulphate to ^{210}Pb can be measured in aerosols collected on a filter using a high-volume air sampler. The sulphate-to- ^{210}Pb ratio in precipitation, when compared with the ratio in aerosols, indicates how much of the sulphur deposited (ultimately measured as sulphate) is not associated with aerosol ^{210}Pb measured on filters. As the bucket collection of precipitation is designed to minimize dry deposition of SO_2 directly in the collector, the excess sulphate is inferred to be due to in-cloud scavenging of SO_2 . In-cloud scavenging so determined is $\sim 30\%$ of the total removal flux. Therefore the additional equation needed for a unique solution for j , c and k , if we assume $^{35}\text{SO}_2$ behaves like ordinary SO_2 , is:

$$cS_0/(kS_p + cS_0) = 0.3 \quad (6)$$

We have used a high-volume air sampler on the roof of Kline Geology Laboratory at Yale to collect SO_2 and aerosol sulphate, and a bucket collector at the same site to determine the wet precipitation flux of ^{35}S . After first retaining the aerosol sulphate on a Teflon-coated glass-fibre filter, SO_2 was collected on a potassium-hydroxide-impregnated filter which was demonstrated to have more than 95% collection efficiency under normal tropospheric conditions¹². (The SO_2/SO_4 ratio in our samples is comparable to the data for sites in ref. 13, indicating that there is no significant uptake of SO_2 on the untreated filter. If there were, the real rate constant for oxidation would become smaller.)

To determine the effective local production rate of ^{35}S , rain-water samples were collected. Immediately after each storm, the rain was passed through an anion-exchange column which had been conditioned with 3M HCl followed by distilled water. The sulphate on the columns was then eluted with 3M HCl followed by distilled water. The eluent was warmed on a hot plate and BaCl_2 solution was added to precipitate BaSO_4 . The precipitate was 'aged' overnight and retained on a glass fibre filter. The total sulphur was determined by gravimetry. Sulphur dioxide gas was released from BaSO_4 by thermal decomposition and its β^- emission from ^{35}S decay was assayed by internal gas counting. A 1.5-litre Oeschger-type counting tube filled with methane was used. As a proportional counter filled with methane can tolerate up to 1% (by volume) of SO_2 without serious quenching effects, the SO_2 content in the counting gas was maintained at less than 0.25%. The sulphur on the KOH-impregnated filter was leached with hydrogen peroxide solution and the leachate then neutralized with 3M HCl to pH = 7. Aerosol sulphate was leached with distilled water. In each case, the solution was passed through an anion-exchange column, and the retained sulphate was then recovered by eluting with 3N HCl followed by distilled water. As for rainwater, we precipitated BaSO_4 and released SO_2 gas in preparation for counting. Table 1 shows the ^{35}S activity of SO_2 , aerosol sulphate and wet precipitation. More than one-third of the ^{35}S in the air is in the form of $^{35}\text{SO}_2$. If any $^{35}\text{SO}_2$ was lost in the first filter because of unknown alkaline aerosols trapped there, this number will be larger. (We do not, however, believe that this is the case for the New Haven area which is not surrounded by a limestone terrain.) The specific activity of ^{35}S in aerosol sulphate was the same as that of gaseous SO_2 , supporting the premise that ^{35}S entered the atmosphere as $^{35}\text{SO}_2$ with little direct 'hot' atom conversion to $^{35}\text{SO}_4$.

We have analysed four precipitation samples from 5 May to 6 June 1990 for ^{35}S . The monthly flux of ^{35}S based on these four samples is ~ 0.022 d.p.m. per cm^2 , a value we assume to be valid for spring and summer. The tropospheric boundary layer receives ^{35}S that is directly produced by cosmic rays throughout the troposphere, but primarily at the higher altitudes and from the stratosphere by tropopausal folding. We designate these sources as the effective local ^{35}S production rate, P , used in equations (1) and (3). As the troposphere contains $\sim 80\%$ of the total atmospheric mass, this deposition rate translates into an effective P_{j+c} for the troposphere of 1.09 d.p.m. ^{35}S per 1,000 m^3 per day. Using the values of S_0 and S_p for an air sample collected on 3 August 1990 and this value of P_{j+c} , we obtain $j = 0.142$ day $^{-1}$, $k = 0.084$ day $^{-1}$ and $c = 0.056$ day $^{-1}$, corresponding

TABLE 1 Contents of total sulphur and ^{35}S produced by cosmic rays in gaseous SO_2 , aerosol sulphate and precipitation at Kline Geology Laboratory

	^{35}S		S
	Specific activity (d.p.m. ^{35}S per mmol S)	Concentration (d.p.m. ^{35}S per 1,000 m^3 air (STP))	($\mu\text{mol S}$ per 1,000 m^3 air (STP))
Air			
3 August, 1990			
Particle S	61.7 $\pm$ 0.9	8.2	133
Gas S	60.2 $\pm$ 0.7	5.3	88
Precipitation			
		(d.p.m. ^{35}S per kg water)	($\mu\text{mol S}$ per kg water)
5 May 1990	105 $\pm$ 1.4	4.0	38
20 May 1990	70 $\pm$ 1.5	2.5	35
28 May 1990	77 $\pm$ 0.9	1.2	17
6 June 1990	53 $\pm$ 0.8	2.1	40

The sulphur concentrations have errors of less than 1% (1σ). The $^{35}\text{S}/\text{S}$ errors are reported as 1σ values. All data are for the Kline Geology Laboratory, Yale University, New Haven, Connecticut, USA.

respectively to a mean SO₂ oxidation time of 7 days, an aerosol mean residence time of 12 days and in-cloud mean scavenging time of 18 days.

This is the first attempt to determine the *in situ* tropospheric SO₂ oxidation rate constant using cosmogenic ³⁵S, and as such it is subject to the uncertainties inherent in novel measurements and oversimplifications. We do, however, have some independent information on the values of *P* and *k* in this region. In 1977 and 1978, Turekian *et al.*¹⁴ determined the deposition flux of ⁷Be (half-life 54 days) in New Haven (again at Kline Geology Laboratory) and obtained a value of 1.88 d.p.m. per cm² per month. The effective 'production' ratio of ⁷Be/³⁵S, as determined from bucket collections, was ~85 (activity ratio), which is close to the value estimated from Lal and Peters¹⁵ and Lal¹⁶. The apparent agreement may be deceptive, however, as dry deposition of SO₂ was presumably not included in the bucket collections, and the determinations of ³⁵S and ⁷Be were made at different times and by different collection methods. The ³⁵S measurements were made on individual storms, whereas the ⁷Be data were obtained on monthly integrated samples collected in a continuously exposed collector. An independent value for *k* was determined for the eastern United States¹⁷ from ²¹⁰Pb and ¹³⁷Cs fluxes measured in soils and atmospheric concentrations, the long-term time-averaged mean was $k \approx 0.2 \pm 0.1$ (2 σ) per day, corresponding to a mean aerosol residence time of $\sim 5 \pm 2.5$ (2 σ) days. Our value of 12 days is somewhat larger than this; the difference may be due to the short-term nature of our observations.

The mean oxidation time of 7 days at the time and location of our experiment is within the range of values predicted for homogeneous reactions¹⁸ and markedly longer than the value based on ³⁸S systematics. The value of about six hours reported by Junkerman and Roedel^{10,19} using ³⁸S is probably due either to hot-atom processes, as suggested by Calvert *et al.*¹¹ or, in our opinion, to surface boundary effects dominated by dry deposition of ³⁸SO₂ on surfaces.

If the time constants for oxidation, in-cloud scavenging and aerosol deposition of ³⁵S are applicable to terrestrial sulphur, as implied by the similarity of the ³⁵S/S ratio of gaseous and particulate sulphur, then air in the boundary layer has efficiently mixed the ³⁵S from above and the terrestrial sulphur horizontally supplied from sources in the boundary layer, at least over a timescale of three days. For the specific time and place, New Haven in August 1990, the values of mean oxidation time and mean aerosol residence time obtained in this work may thus be valid for all sources of SO₂ entering the boundary layer. We expect these time constants to differ both with climate and location. Our method may provide a way of measuring the variation with position and time with a suitable deployment of collectors.

The role of dry deposition in removing SO₂ could also be determined by comparing the ³⁵S fluxes from bucket collections of precipitation with those from integrated vegetative and soil profiles, the difference between these yielding the ³⁵S flux to the ground surface by dry deposition. We are at present undertaking such a study. □

Received 29 January; accepted 29 May 1991.

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ACKNOWLEDGEMENTS. We thank P. Parker and M. Wahlen for help with our low-level internal gas counting system and P. Crutzen for helping to improve the manuscript. This study was supported by the US Department of Energy and the NSF.

Influence of biomass burning on equatorial African rains

Hélène Cachier & Joëlle Ducret

Centre des Faibles Radioactivités, CNRS-CEA, Avenue de la terrasse, 91198 Gif-sur-Yvette, France

BIOMASS burning affects the African continent all year round^{1–4}. In the dry season there are widespread savannah fires, and there are always some domestic and agricultural fires. Here we present measurement of particulate black carbon, which is an unambiguous indicator of combustion, in rain waters collected at a remote site in the Northern Congo. We find that the rains contain pollutants from biomass burning, and are particularly affected during the Northern Hemisphere dry season. Our results show that biomass-burning smoke particles may act as cloud condensation nuclei, and might thereby play a part in cloud formation and hence precipitation in the tropics³.

Forty-six rain samples were collected over one year (June 1988–June 1989) at Enyele (2°5' N, 18° E) in the primary forest of Northern Congo. The equatorial forest, which is embedded between two large and populated savannah areas located north and south of the equator, is likely to be influenced by the biomass burning practices. Our sampling site is located in the dense part of the remaining virgin forest of Africa and almost unaffected by local human activities. It has an equatorial climate, with abundant rainfall all year round. A whole year's sampling of rain in this region can be considered to register the annually repeated influence of anthropogenic activities in both the northern and southern savannah regions of the African continent. Precipitation was collected as it occurred by an automatic rain collector. Rain water was then rapidly filtered on pre-cleaned glass-fibre filters. We determined total carbon (C_t) and black carbon (C_b) contents from halves of the same filter, following an analytical protocol previously reported for aerosols^{2,5}.

As shown in Fig. 1, black carbon is present in all samples, which unambiguously indicates that combustion inputs influence equatorial rains all year round. In tropical areas, the main combustion activity is likely to be the burning of biomass^{1,6}. The overwhelming influence of vegetation combustion on tropical rains in Africa has already been suggested by Lacaux *et al.*^{7,8} who measured low pH values in rains collected in the Ivory Coast and Congo, mainly due to high contents of NO₃[−] ions which they attributed primarily to biomass burning inputs. In our rain samples too, the pH is low, with a mean value of ~4.4 (B. Lefeuvre and J. P. Lacaux, personal communication), which is similar to values reported in different tropical or equatorial regions^{7–10}. Mean values obtained for black and total carbon contents are 73 and 292 µg C l^{−1} respectively (Table 1). The few data available from previous work suggest that the particulate carbon content of our rain samples is similar to those found in temperate source regions^{11,12}, although our sampling site is a hundred kilometres from the savannah source regions. This result indicates the importance of the particulate inputs from biomass burning and their long-range transport over the African continent. Figure 1 also stresses the predominance of the organic mode, which accounts for ~75% of the total carbon concentration found in the particulate phase in the rains. We

recall our previous studies on tropical carbonaceous aerosols^{13,14}, indicating that the biomass burning processes produce an organic-rich aerosol, which probably reflects poorly oxygenated combustions. Thus, the chief part of the particulate organic content in rains could also originate from the burning of the biomass. The concentrations of total and black carbon peak simultaneously, which reinforces the hypothesis of a common origin for the two carbonaceous components (C_b and C_o , which is assumed to be $C_t - C_b$) of the rain particles.

Consideration of the large-scale circulation pattern repetitively prevailing over Africa throughout the year can provide information on possible atmospheric inputs from biomass burning at our study site. In Africa, seasons are determined by the displacement of two discontinuity surfaces, separating hot, dry continental air masses from cooler and more humid maritime ones¹⁵⁻¹⁷: the intertropical convergence zone (ITCZ) is formed by the convergence of Sahelian and equatorial ocean air masses; the other is the interoceanic confluence (IOC) which separates air coming from the Southern Atlantic from that of the Indian Ocean. The extreme positions of these frontiers occur in January and July, which corresponds to the heart of the dry season in the Northern Hemisphere and Southern Hemisphere respectively. These positions are shown in Fig. 2a. During the dry season, biomass burning is at a maximum. For the savannahs of the Northern Hemisphere, the dry season is established once the ITCZ has moved south of these regions. A few weeks after the beginning of the dry season, savannah fires are widespread and last about a month. The sweeping of the ITCZ thus induces a latitudinal shift of the regions affected by savannah bushfires. It may be considered that the duration of the fire season in the whole African savannah region of the Northern Hemisphere is 3-4 months¹, centred on January. At the same time, the shifting of the IOC generates the sequence of seasons in the Southern Hemisphere, the regions having the dry season being those then located southeast of the IOC. The burning season peaks in July and lasts 4-5 months¹.

But the ground location of these convergence zones alone is not sufficient to explain the presence of biomass-burning pollutants at Enyele. At ground level, the sampling site always seems to be in the atlantic monsoon regime and therefore not affected by atmospheric fluxes coming from the savannah regions (Fig. 2a). The influence of biomass burning in rains at Enyele can be seen, however, by considering the vertical profiles of the ITCZ^{15,16} and the IOC¹⁶, which both show a shift with altitude. Figure 2b shows that areas directly affected by continental inputs during the dry season are much greater than previously suggested. In particular, during certain periods of the year, the forested equatorial regions of Central Africa, where the extreme locations of the convergence zones are observed, may be influenced by high-layer air masses that are built up in the savannah regions. These considerations indicate that because of the favourable geographical position of the sampling site and the alternation of savannah regions that are in the dry season, rains falling over Enyele are influenced during much of the year by

TABLE 1 Mean concentrations of total and black particulate carbon in rains

	C_o ($\mu\text{g C l}^{-1}$)	C_t ($\mu\text{g C l}^{-1}$)	C_b/C_t (%)
Seattle*	60		
Sweden*	100		
Paris region†	335 ± 320	1,185 ± 1,190	29 ± 10
Enyele‡			
Whole year	73 ± 59	292 ± 162	24 ± 10
November–March	155 ± 59	477 ± 173	33 ± 6
Rest of the year	45 ± 18	236 ± 89	20 ± 6

* Data from Ogren *et al.*¹¹.

† Data from Ducret and Cachier¹².

‡ This work collected June 1988–June 1989.

severe savannah fires, raging either in the Northern or in the Southern Hemisphere.

In Fig. 1, the group of rains collected during the dry season of the Northern Hemisphere (from November to March) is clearly separate from the others, with both particulate total and black carbon concentrations two or three times as great as during the rest of the year (Table 1). This feature is not affected by the variation of the seasonal amount of rainfall (mean amount of rain from November to March was 16 mm; for the other months it was 22 mm). Our results do not, however, clearly record the other main expected episode of biomass burning. That is, the start of the bushfire season in the Southern Hemisphere is not clearly reflected by an increase in the rain particulate concentrations. This may either be due to differences in the intensity and frequency of biomass burning in the African northern and southern savannahs (the northern countries supporting a larger population) or to the geographical position being more favourable for the efficient atmospheric transport of continental emissions from the northern regions. Another salient point of our data is the occurrence of a background combustion component in rain samples collected during periods when savannah bushfires are limited (October–November, April–May). This background of pollution cannot be attributed to local or even regional activities. It confirms the continuous influence throughout the year of biomass-burning emissions, primarily through domestic and agricultural fires (use of wood and charcoal for cooking, slash-and-burn fires), and the complexity of atmospheric transport and deposition patterns over this region of the African continent.

Preliminary calculations based on recent estimates of forested areas¹, rainfall in equatorial regions (2.2 m)¹, areas of burnt savannah⁴ and fine aerosol emission factors in biomass combustions², and on our rain concentration data, indicate that ~15% of the 2 tg of black carbon released in the atmosphere of Africa, essentially by savannah fires, is removed by wet deposition over the whole humid African forest. The primary forest of Northern

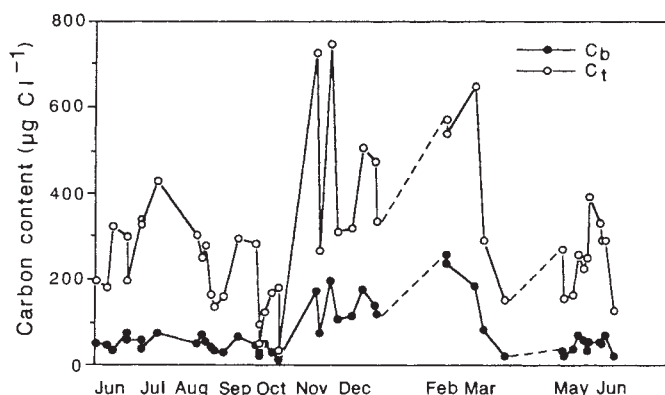


FIG. 1 Black carbon and total carbon contents of rains over the primary forest of Enyele (Northern Congo).

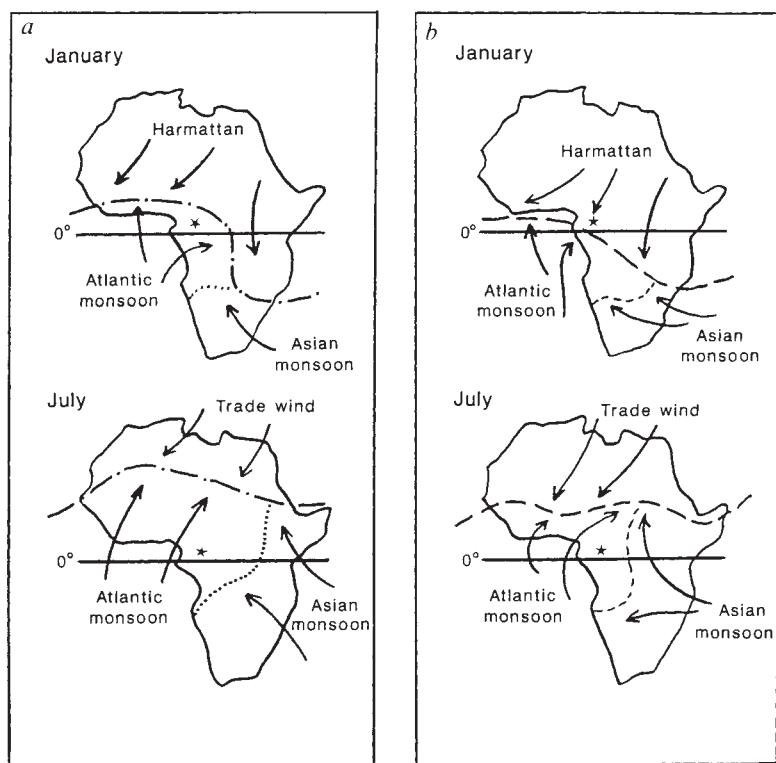


FIG. 2 *a*, Ground location of the two discontinuity surfaces ITCZ (---) and IOC (····) in January and in July. *b*, Position of ITCZ (---) and IOC (---) at pressure level of 850 mbar for the same period. ★, location of the sampling site.

Congo itself could receive more than 10% of these inputs.

Previous work^{13,14} has indicated that the fluctuations of the relative abundance of black carbon (expressed as C_b/C_t) recorded in the aerosols could be related to different combustion processes, depending on the type of vegetation being burnt and on the physical parameters of the fire. An important result was the noticeable increase of the black carbon content in aerosols generated by well oxygenated large-scale bushfires. Applying such an approach to our rain sample results shows that the apparent covariation of the total and the black carbon contents hides important fluctuations of the C_b/C_t ratio, which ranges between 10 and 57%. Such variations of the black carbon content in rains might indicate differences in the biomass-burning sources, as already seen for aerosols. From the C_b/C_t ratio it can also be seen that the group of rain samples collected from November to March (the dry season in the Northern Hemisphere), is separate from the others, with a mean value of 35% which is significantly different from that obtained for the other samples (mean value of 20%). This result suggests that during this period, the main combustion sources influencing the rains are different from those affecting rains during the rest of the year. The higher C_b/C_t ratios in these November–March samples could reflect inputs of products emitted by more oxygenated fires, namely the large-scale savannah fires of the Northern Hemisphere.

It must be emphasized that the black carbon fraction is more abundant in our rain samples (mean $C_b/C_t = 24\%$) than in the tropical aerosol samples in source regions (mean $C_b/C_t = 12\%$)^{13,14}. This apparent discrepancy suggests that a considerable fraction of the organic aerosol dissolves in the hydrometeors, enhancing the relative importance of black carbon in the particulate phase. It may be hypothesized that the hydrophilic behaviour of carbonaceous aerosols from biomass burning is due to the coating of particles by water-soluble material. This coating could be gained rapidly in the biomass-burning plume because of the combination of two main factors: the surface properties of the carbonaceous particles which are efficient absorbants of gases, and the enrichment of the plume in mineral (such as HNO_3 and NH_3) and gaseous organic species. As the

biomass-burning aerosols are mainly submicrometre-sized particles, they are potential nuclei for cloud condensation^{3,18}. The partial disappearance of organics from the particulate phase in rain waters may indicate that the carbonaceous particles are incorporated in the hydrometeors, and it could be a key step in enabling these aerosols to act as cloud condensation nuclei. If so, our rain concentration data indicate that the important production of particles by biomass combustion could alter the radiative balance and the precipitation regimes in the Tropics. Finally, the question of whether the partial dissolution of organic aerosols produced by biomass burning contributes to the dissolved organic carbon load and the organic acidity of the present or previous rains deserves further investigation. □

Received 12 February; accepted 28 May 1991.

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ACKNOWLEDGEMENTS. We thank P. Buat-Ménard, B. Cros, R. Delmas and J. P. Lacaux for helpful comments. This work was supported by the CNRS and the CEA, and the PIREN-CNRS and the Ministère de l'Environnement in France (DECAFE Program). We thank the University of Brazzaville (Congo) for logistic support.

Lower-mantle structure from ScS–S differential travel times

Robert L. Woodward* & Guy Masters

Institute of Geophysics and Planetary Physics,
Scripps Institution of Oceanography, University of California, San Diego,
La Jolla, California 92093, USA

* Present address: Department of Earth and Planetary Sciences,
Harvard University, 20 Oxford Street, Cambridge,
Massachusetts 02138, USA

KNOWLEDGE of the spectrum of structure in the lower mantle is crucial to our understanding of the dynamical evolution of the Earth and, in principle, can be inferred from the analysis of global seismic data sets. The long-wavelength compressional velocity structure has usually been constrained through tomographic inversion of the ISC catalogue of *P*-wave travel times^{1–3}, whereas shear velocity has been inferred from waveform modelling of long-period shear waves^{4–6}. The models that have been produced are similar in only the largest-scale features⁷ and explain little of the variance of the raw data used in their construction. Thus, there is a legitimate concern that the current generation of global-scale models give only a crude approximation to the largest-scale structure and that there may be significant aliasing of short-wavelength heterogeneity. Here we use ScS–S differential travel times to demonstrate that the three-dimensional structure of the lower mantle is indeed dominated by continental-scale features. Particularly convincing is the fact that the features are apparent in the raw data and are not the product of a complicated modelling procedure

ScS–S differential travel times are ideal for investigating lower-mantle structure. They have almost no sensitivity to structure in the upper mantle because, for large epicentral distances, their ray paths are nearly identical there (Fig. 1a). They are relatively insensitive to errors in source location, with reasonable variations in hypocentral parameters leading to errors in ScS–S times of less than a second. Finally, the differential time of ScS relative to S can readily be measured to a precision of better than 1 s by cross-correlation of waveforms⁸.

All our measurements were made from transverse-component long-period GDSN (global digital seismograph network) seismograms. We were restricted to a distance range of 45°–75° where ScS is usually a clearly identifiable arrival (Fig. 1b) and there is no interference of ScS with the S and SS arrivals. We used the transverse component to minimize contamination from converted phases and chose the long-period channels to minim-

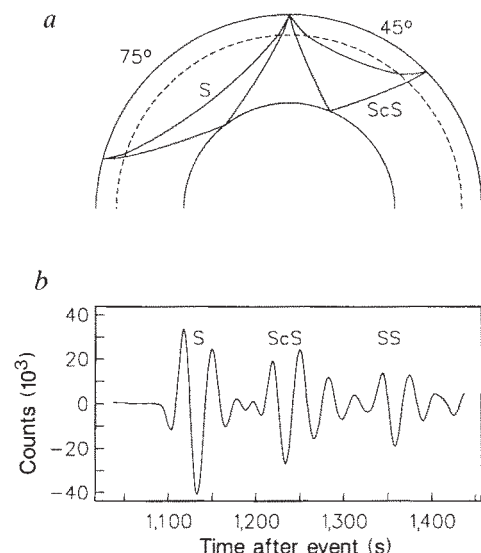
ize the possibility of spatial aliasing from short wavelength structure. The instrument responses of the long-period GDSN have pass-bands centred at ~25 s so that the characteristic wavelength of the S body waves is ~200 km. In reality, the data smooth structure in a complicated way over length scales greater than a wavelength. For example, a Fresnel zone calculation suggests that long-period ScS reflects coherently from a patch of the core/mantle boundary which is ~5° (300 km) in diameter, and we interpret this calculation as a rough measure of the potential resolution of the data.

We inspected seismograms in the distance range of interest for all events with body-wave magnitude $m_b \geq 5.5$ recorded by the GDSN in the years 1976–86. More than 2,600 measurements were obtained from a total of 1,383 events recorded at 45 globally distributed stations, giving a homogeneous dataset with reasonable global coverage. Residuals were calculated relative to the isotropic PREM model⁹ and corrected for ellipticity¹⁰. The mean of the residuals shows almost no distance dependence and is effectively zero, implying that the measurements are, on average, consistent with the lower-mantle shear velocity given by PREM.

Figure 2a shows the distribution of ScS–S measurements plotted at the surface projections of ScS bounce points. Good coverage is obtained in the circum-Pacific region, but coverage in other areas is sparse because of source–receiver distribution and the limited epicentral distance range in which S and ScS can be identified clearly. The residuals show a remarkable large-scale coherence. These patterns are even more apparent in Fig. 2b, which shows the residuals after the application of what is effectively a running-mean smoothing procedure⁸. The smoothing is relatively light, averaging over length scales of ~1,000 km, and makes the patterns in the heavily sampled areas much clearer. We have characterized the observed pattern by using spherical splines to fit a smooth splining function to the data^{11,12}. Subtraction of the smooth splining function from the individual observations revealed a few outliers, although only 44 of 2,649 initial measurements had to be discarded, indicating that the data set is internally consistent. After removal of outliers, the distribution of residuals has a SMAD (scaled median absolute deviation)¹³, a robust estimate of the width of the distribution) of 2.9 s, although residuals greater than 10 s are occasionally observed. The splining function can easily be expanded in spherical harmonics, revealing that its power is concentrated in harmonic degrees 1–3, with most of the signal in degree 2. This provides quantitative confirmation of the extremely long-wavelength nature of the pattern shown in Fig. 2a and b.

We would like to know the location of the anomaly that causes the observed residual pattern. As noted above, upper-mantle structure should not contribute significantly to the differential

FIG. 1 a, Diagram of the ray paths of S and ScS at epicentral distances of 45° and 75°. The ScS phase is reflected at the core–mantle boundary while, at these distances, the S phase is turning in the lower mantle. The surface and core–mantle boundary are shown by solid line semi-circles, and the dashed-line semi-circle is drawn at a depth of 670 km. Note how the ray paths of S and ScS are very close in the upper mantle. Thus, ScS–S times are nearly insensitive to structure in the upper mantle. b, Typical long-period seismogram from the GDSN, showing arrivals of the phases S, ScS and SS. Recording is from station GUMO at 59° epicentral distance from a 31-km deep event.



times. To test this, we have calculated theoretical ScS–S times for our ray geometry using the upper-mantle model M84C¹⁴ and the upper-mantle portion of model MDLSH⁶. The SMADs of the distributions of residuals predicted by these two models are both 0.25 s, which is much smaller than the 2.9 s found in the data. The topography of the core–mantle boundary could be the source of some of the signal in the data, although current models tend to have topography restricted to less than 5 km in amplitude, which would explain little of our observations. For example, the topography model of Morelli and Dziewonski¹⁵ predicts a distribution of residuals with a SMAD of only 0.2 s. Thus the observed signal is due to velocity anomalies in the

lower mantle, either near the S turning point at a depth of ~1,500 km or near the ScS bounce point in the lowermost mantle.

In fact, the location of the structure causing the anomaly in differential travel time in the lower mantle seems to vary geographically. Some studies have obtained a good correlation between absolute S times and ScS–S residuals for measurements with bounce points beneath the Caribbean^{16,17}. This correlation suggests that the travel-time anomaly originates in the S leg in the mid-mantle, and such an anomaly has also been imaged in tomographic studies¹⁸. In contrast, other workers¹⁹ see a correlation between ScS residuals and ScS–S residuals

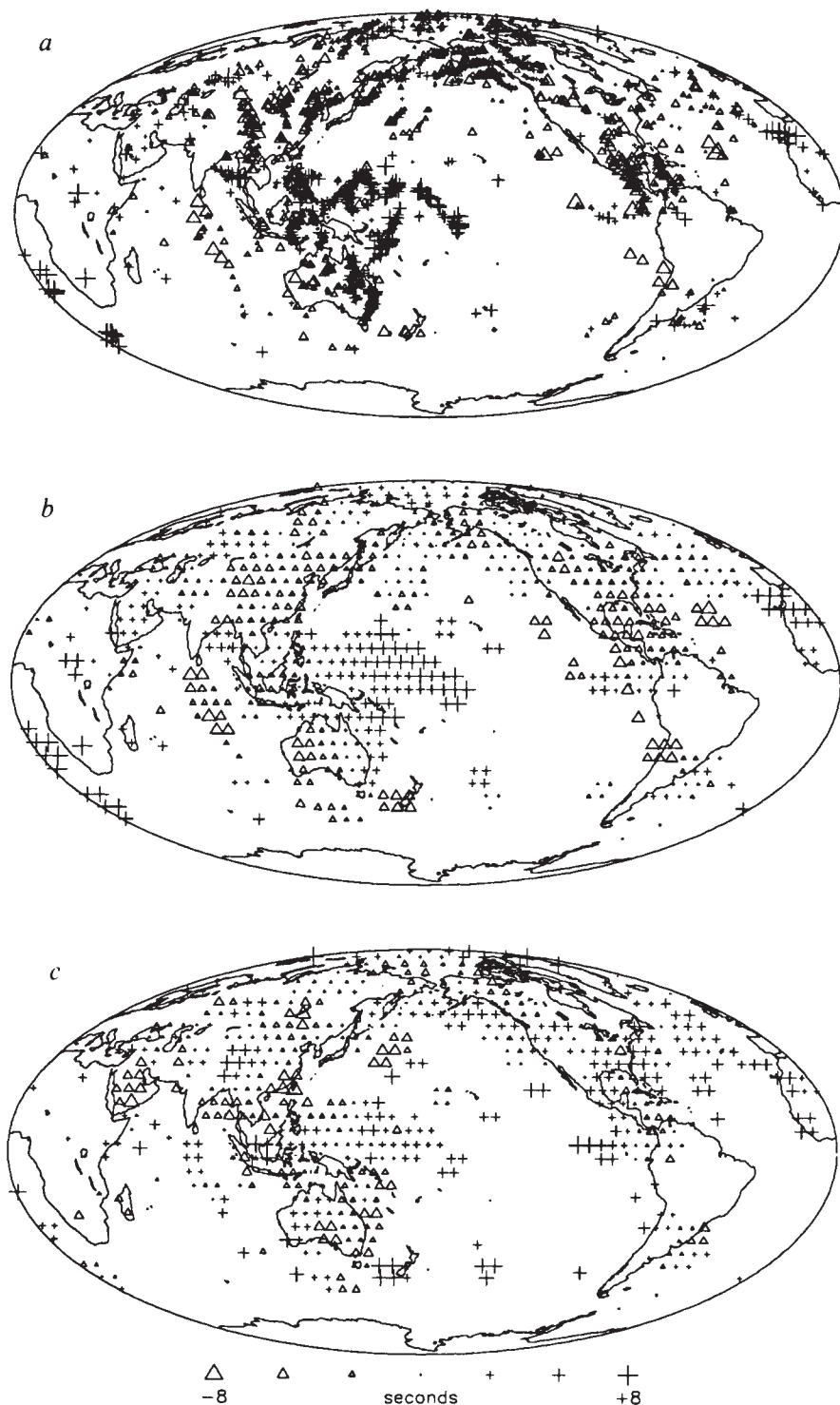


FIG. 2 *a*, The observed ScS–S residuals, plotted on a Mollweide equal-area projection. Symbols are plotted at the surface projections of the ScS bounce points. Triangles indicate negative residuals and pluses indicate positive residuals. The scale bar for all three maps is at the bottom of the figure. Symbol heights increase linearly with residual size. Note that a negative residual can mean either that ScS is fast and so arrives early or that S is slow and arrives late. The data show remarkable large-scale consistency. *b*, The residuals shown in *a* after being lightly smoothed by a running-mean smoothing procedure. In addition to the smoothed values, this map contains 22 individual measurements which did not have enough nearby measurements to be included in the smoothing process. The large-scale patterns are obvious after smoothing, particularly in the heavily sampled areas. *c*, Observed S residuals, after application of the running-mean smoothing (including 19 individual observations which were not included in the smoothing process, because of lack of nearby measurements). Symbols are plotted at the surface projections of the S turning points. The residuals have been corrected for ellipticity and their mean removed. Although these measurements are susceptible to imprinting from upper-mantle structure and from location errors, large-scale patterns are discernible.

for measurements in the North Atlantic and Indian Oceans, suggesting the anomaly originates in the ScS leg at depths below 2,000 km.

To determine whether most of the observed signal is accumulated on the ScS leg or the S leg, we measured absolute S travel times by correlation of the S phase with a synthetic seismogram. Our measurement procedure consisted of cross-correlating the first half-cycle of the S phase (which is not contaminated by depth phases for events deeper than 20 km) with a synthetic seismogram which was created by convolving a delta function with the instrument response and an attenuation operator. We generally obtained very good fits to the data, and nearly 2,000 of the 2,605 recordings that yielded ScS–S measurements gave well-constrained S times. These absolute S measurements are intrinsically less precise than the differential times and are more susceptible to location and timing errors. Errors in the absorption-band operator and model-dependent location biases lead to systematic distance and event-depth dependencies in the data. These background trends have been assessed using a much larger dataset of absolute S times and have been removed before comparison with the ScS–S data. The S residuals in the distance range of interest show a coherent geographic pattern when plotted at the turning point of S (Fig. 2c) although this pattern does not correlate well with the pattern of ScS–S. The scatter plots of S against ScS–S and ScS against ScS–S (Fig. 3) demonstrate this. The strong correlation of ScS with ScS–S shows that, in a global sense, the differential time anomaly is accumulated along the ScS leg. This result agrees with many other studies which have found enhanced levels of heterogeneity near the base of the mantle^{1,6,20}.

As the magnitude of absolute S and ScS travel-time residuals shown in Fig. 3 indicates that there is considerable heterogeneity in both the mid- and lowermost-mantle, a straightforward interpretation of the pattern in the data is not possible. We have therefore tested some current models of lower-mantle structure

to see if they can explain the data. Most models contain a ring of high velocities around the Pacific in the lower mantle, which produces a residual pattern qualitatively similar to that seen in the data although quantitative measures of pattern correlation show that no current model provides an acceptable fit to the data. In particular, models that do not have large-amplitude structure in the lowermost mantle (such as MDLSH⁶) cannot reproduce the correlation plots of Fig. 3.

Our most important result is the demonstration that ScS–S residuals show coherent, continental-scale patterns when plotted at the bounce point of ScS. These patterns are reminiscent of those seen in our studies of SS–S. Indeed, the amplitude of the differential ScS–S patterns is not much smaller than that induced in SS–S times by continent and ocean structure at the surface⁸. The harmonic analysis, showing that the power in the pattern seen in the ScS–S data is concentrated in harmonic degree 2, is in qualitative agreement with observations of splitting of normal modes which sample the lower mantle^{21–23}. The fact that degree-2 structure seems to dominate throughout the mantle has also been emphasized in other studies⁶.

We have compared the ScS–S data with the predictions of models of lower-mantle S-wave structure. Although no model gives a completely acceptable fit to the ScS–S data, models with large amplitude structure in the lowermost mantle best reproduce the characteristics of our dataset. It is probable that a satisfactory model could be built which would have 1–2% anomalies in the mid-mantle increasing to 3–5% in the lowermost mantle. This size of anomaly for the lowermost mantle has been suggested by studies using many different kinds of seismic data^{24–27} and is similar in magnitude to the large-scale anomalies seen near the surface. It is probable that chemical variations would be required to explain such lateral variations, providing support for the idea of 'continents' on the core-mantle boundary²⁸.

Finally, the fact that there is such large-amplitude, large-scale structure in the lower mantle can affect the interpretation of other seismological data sets. For example, ScS₂–ScS times have been interpreted in terms of upper-mantle structure alone²⁹. As it is likely that such data can map lower-mantle structure into the upper mantle, a careful re-evaluation of the ScS data might be advisable. □

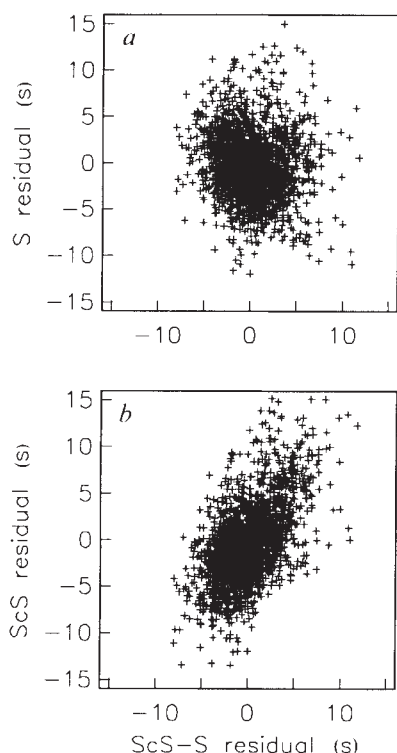


FIG. 3 *a*, S against ScS–S residuals. *b*, ScS against ScS–S residuals. The correlation of ScS with ScS–S indicates that most of the time anomaly in ScS–S is accumulated along the ScS leg, although the large scatter indicates that a considerable fraction of the time anomaly is accumulated in the mid-mantle.

Received 16 January; accepted 30 May 1991.

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ACKNOWLEDGEMENTS. S. Mercer contributed greatly at the beginning of this project and is responsible for some of the ScS–S picks. P. Shearer performed the Fresnel zone calculation for ScS. This research was supported by the NSF.

Fish-like gills and breathing in the earliest known tetrapod

M. I. Coates & J. A. Clack

University Museum of Zoology, Downing Street, Cambridge CB4 3EJ, UK

THE origin of tetrapods is generally associated with the emergence of terrestrial vertebrate life. Anatomical features unique to tetrapods are usually considered to be adaptations to the terrestrial environment. Here we report the discovery of a fish-like branchial skeleton in *Acanthostega gunnari*, from the Upper Devonian of East Greenland, one of the earliest tetrapods known. It shows a proximally expanded ceratohyal and large, ventrally grooved ceratobranchials. Such grooves are found in the ceratobranchials of modern fishes, and house the afferent branchial aortic arches. The shoulder girdle bears a postbranchial lamina along its anterior margin. In fishes this supports the posterior wall of the opercular chamber. *Acanthostega* seems to have retained fish-like internal gills and an open opercular chamber for use in aquatic respiration, implying that the earliest tetrapods were not fully terrestrial. The discovery provides information on the sequence of acquisition of tetrapod characters, and supports previous suggestions that such characters as legs with digits¹ evolved first for use in water.

The material of *Acanthostega gunnari* was found in 1987 by a Cambridge-Copenhagen expedition². Most of the information on the branchial skeleton has come from an almost complete articulated specimen MGUH (Geology Museum, University of Copenhagen), field number 1227, which has already yielded the earliest-known tetrapod stapes³ and an eight-digitated forelimb¹. Other material yielding hyobranchial elements includes UMCZ (University Museum of Zoology, Cambridge) T 1300 and MGUH 1258 and 1300. Associated with MGUH 1227 are six or seven ceratobranchial elements, the right ceratohyal and less certainly two hypohyals (Fig. 1). Most lie almost in the expected life position, although the right ceratohyal has been rotated by 180° and lies on the left side. An incomplete left ceratohyal is known from specimen MGUH field number 1300 (Fig. 2b), and Fig. 2a shows a reconstructed left ceratohyal in lateral view.

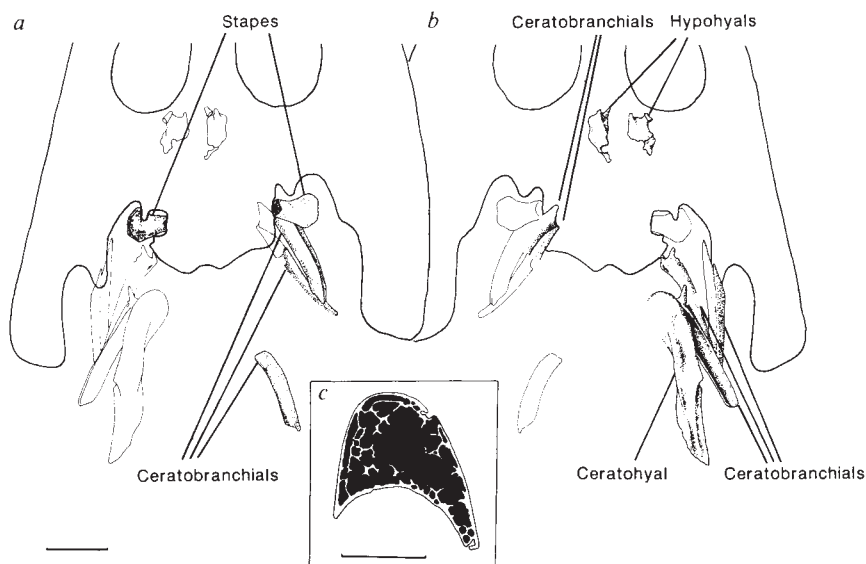
The best preserved ceratobranchial is bifurcated anteriorly for articulation with the basibranchial and, where exposed, the elements bear deep grooves along their ventral surfaces (Fig. 1c). Such grooves resemble those in the ceratobranchials of most bony fishes. The ceratohyal is expanded proximally (posteriorly) into a blade with a rounded end bearing a strong ridge on its

lateral surface, whereas distally it is narrower and grooved. The ceratohyal, and other known elements of the hyoid arch, most closely resemble those of a Devonian lungfish such as *Chirodipterus*⁴ rather than the proximally narrow, bipartite ceratohyal and large hyomandibula of the Devonian osteolepiform *Eusthenopteron*⁵.

This branchial skeleton is more fish-like than that of any other known tetrapod. Some temnospondyl specimens, the group of largely Palaeozoic amphibians which is thought to have given rise to frogs and probably urodeles⁶, preserve some of the branchial skeleton. In *Dvinosaurus* the ceratobranchials are stout blunt-ended elements, bearing faint grooves on the ventral surface, interpreted as showing that this animal was perenni-branchiate, with external gills^{7,8}. The ceratobranchials of *Acanthostega* are much more deeply grooved and more thoroughly ossified; other early tetrapod ceratobranchials are short, suggesting incomplete ossification of the gill skeleton (see also those of the actinopterygian *Psephurus gladius*⁹). In modern fishes, such grooves are associated with the possession of paired gill filaments, and carry the afferent branchial aortic arches. The most gill-dependent of the living lungfishes, *Neoceratodus*¹⁰, has cartilaginous ceratobranchials which resemble those of *Acanthostega* (M.I.C., unpublished observation). Morphologically similar and fully ossified gill skeletons are found in *Polypterus*¹¹, the fossil gill-breathing lungfish *Chirodipterus*^{4,10}, and *Eusthenopteron*⁵.

Further fish-like characters are found in the shoulder girdle (specimens MGUH 1258, 1227) (Fig. 3). The cleithrum is a large dermal element in *Acanthostega*, comparable only to that of *Ichthyostega* among tetrapods. The scapular blade is co-ossified with the cleithrum and its extent is uncertain, but there is no supraglenoid foramen. The anterior edge of the cleithrum carries a distinct anteroventral process which clasped the dorsal process of the clavicle. This character is unique to *Acanthostega* among tetrapods, but shared with Palaeozoic sarcopterygians such as the lungfish *Scaumenacia*⁵ and the osteolepiform *Eusthenopteron*⁵. The cleithrum bears a smooth postbranchial lamina similar to that found in gill-breathing bony fishes, where it forms the posterior wall of the open opercular chamber. *Acanthostega* retains an anocleithrum, part of the supracleithral series of bones, generally lost in tetrapods. *Tulerpeton*, from the Upper Devonian of Russia¹², is the only other tetrapod known to retain an anocleithrum. In *Acanthostega* it is a large ovoid bone articulating with the dorsal end of the cleithrum mesially, and known from both isolated and articulated specimens. It resembles that of the extant lungfish *Neoceratodus*⁵.

FIG. 1 *Acanthostega gunnari*. a, Dorsal view of branchial skeleton (MGUH 1227). Thick outer line indicates perimeter of postorbital region of skull. Outlines indicate relative position of branchial skeleton revealed on ventral surface. b, Ventral view of branchial skeleton (MGUH 1227). Diagram follows same convention as in a. Scale bar, 10 mm. c, Cross-section through ceratobranchial (UMCZ T 1300). White areas are preserved bone; black area is matrix infill of endochondral spaces. Scale bar, 2 mm.



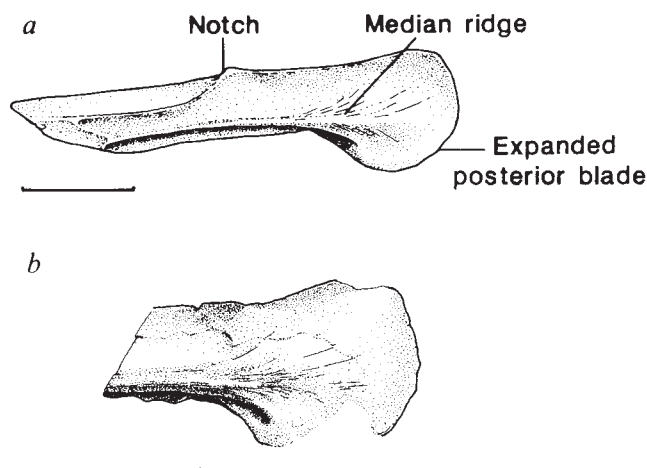


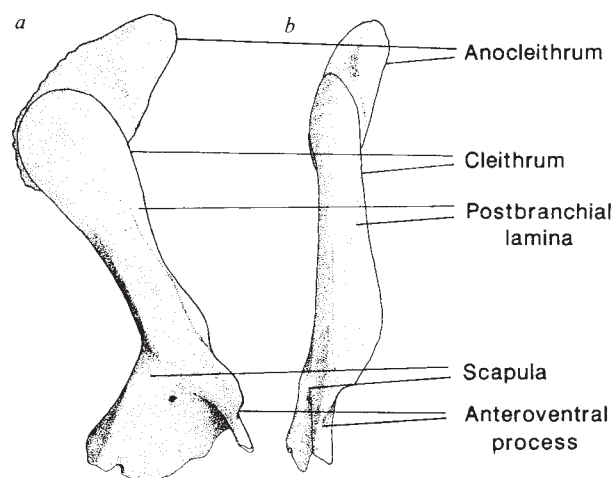
FIG. 3 *Acanthostega gunnari*. Right pectoral girdle (incomplete ventrally) (MGUH 1258), showing articulation between cleithral blade and anocleithrum, broad postbranchial lamina, and anteroventral process. a, Lateral view; b, anterior view. Scale bar, 10 mm.

Many modern tetrapods retain the branchial skeleton, though it is not used to support respiratory internal gills. The unossified branchial skeleton of the urodeles resembles most closely the fish condition. In urodeles it supports the tongue musculature and the floor of the buccal cavity, moving it during buccal ventilation¹³. Larval and perennibranchiate amphibians retain an open operculum and gill clefts supported by the branchial skeleton. The branchial arches are not grooved but oval in section, and the shoulder girdle makes no contribution to the posterior wall of the opercular chamber. There are only external gills and no paired filaments (M.I.C. and J.A.C., unpublished observation). Specimen MGUH 1227 was not apparently full-grown, having a skull and humerus about one-third smaller than the largest known individuals; however, the gill skeleton, phalanges and tarsus were well ossified and the dermal ornament well developed. There is no evidence that this individual was premetamorphic or paedomorphic.

Acanthostega combines characters of the gill skeleton and shoulder girdle that are associated in fishes with functional internal gills. The morphology is most parsimoniously explained by the suggestion that *Acanthostega* also possessed functional internal gills, a primitive retention unknown in other tetrapods. It is generally believed that possession of lungs is a primitive character in osteichthyan fishes, and they are found in the actinopterygian *Polypterus* and lungfishes. *Acanthostega* probably also possessed lungs, ventilated by movements of buccal pumping involving the hyoid arch³ as in these fishes. Like them, it probably used both modes of gas exchange in appropriate circumstances.

Retention of fish-like internal gills by a Devonian tetrapod blurs the traditional distinction between tetrapods and fishes¹⁴.

FIG. 2 *Acanthostega gunnari*. a, Lateral view of reconstructed left ceratohyal, anterior to left of plate. b, Incomplete posterior portion of left ceratohyal, UMZC T 1300. Scale bar, 10 mm.



It shows that the opercular, submandibular and gular series of bones were lost from the skull while the internal gills were still functional. It implies that metamorphosis was a less profound process in this early tetrapod than in modern amphibians; probably restricted to loss of external gills, if any. The form of this gill skeleton and shoulder girdle suggests that *Acanthostega* was not an obligate air-breather, that it was primarily aquatic and did not derive from an even earlier but more terrestrial tetrapod. Alternatively, the ossified, deeply grooved ceratobranchials may be interpreted as having borne only external gills, or representing a retained larval morphology no longer associated with gills but incorporated into a muscular buccal ventilation system. Similarly, the postbranchial lamina may be a primitive feature of gill-less early tetrapods, unmodified from the fish condition. But none of these alternative interpretations can be found among extant vertebrates. *Acanthostega* resembles gill-breathing lungfishes: both show increased contact between the palate and braincase associated with loss of the operculogular series, reduction of the upper half of the hyoid arch (hyomandibula/stapes), proximal expansion and growth of the lower half (ceratohyal), retention of a substantial grooved gill skeleton and a pectoral girdle with a broad postbranchial lamina^{4,10}. This adds further support to the suggestion that unique tetrapod characters such as limbs with digits evolved first for use in water^{1,15} rather than for walking on land. □

Received 17 April; accepted 7 June 1991.

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ACKNOWLEDGEMENTS. We thank S. E. Bendix-Almgren and the staff of the Greenland Geological Survey for organizing the expedition to collect the material and for permission to work on the specimens, and S. M. Finney for preparing the material.

Better speech recognition with cochlear implants

Blake S. Wilson^{*†}, Charles C. Finley^{*†},
Dewey T. Lawson^{*}, Robert D. Wolford[‡],
Donald K. Eddington[§] & William M. Rabinowitz[§]

^{*}Neuroscience Program, Research Triangle Institute,
Research Triangle Park, North Carolina 27709, USA

[†]Division of Otolaryngology and [‡]Center for Speech and
Hearing Disorders, Duke University Medical Center, Durham,
North Carolina 27710, USA

[§]Research Laboratory of Electronics, Massachusetts Institute of
Technology, Cambridge, Massachusetts 02139, Cochlear Implant Research
Laboratory, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts
02114, and Department of Otology and Laryngology, Harvard Medical
School, Boston, Massachusetts 02115, USA

HIGH levels of speech recognition have been achieved with a new sound processing strategy for multielectrode cochlear implants. A cochlear implant system consists of one or more implanted electrodes for direct electrical activation of the auditory nerve, an external speech processor that transforms a microphone input into stimuli for each electrode, and a transcutaneous (rf-link) or percutaneous (direct) connection between the processor and the electrodes. We report here the comparison of the new strategy and a standard clinical processor. The standard compressed analogue (CA) processor^{1,2} presented analogue waveforms simultaneously to all electrodes, whereas the new continuous interleaved sampling

(CIS) strategy presented brief pulses to each electrode in a nonoverlapping sequence. Seven experienced implant users, selected for their excellent performance with the CA processor, participated as subjects. The new strategy produced large improvements in the scores of speech reception tests for all subjects. These results have important implications for the treatment of deafness and for minimal representations of speech at the auditory periphery.

Features of the two strategies are illustrated in Figs 1 and 2. Both strategies use multiple monopolar electrodes to mimic crudely the tonotopic or 'place' coding of frequencies in the normal cochlea. In particular, high-frequency sounds are indicated by stimulating electrodes toward the base of the cochlea, whereas low-frequency sounds are indicated by stimulating electrodes closer to the apex.

In the CA strategy, microphone signals varying over a wide dynamic range are compressed to the narrow dynamic range of electrically evoked hearing^{3,4} using an automatic gain control (AGC). The AGC output is filtered into four contiguous frequency bands for presentation to each of four electrodes. Information about speech sounds is contained in the relative stimulus amplitudes among the four electrode channels and in the temporal details of the waveforms for each channel (Fig. 2b). A concern associated with this method of presenting information is that only part of it can be perceived by implant patients⁵. For example, most patients cannot perceive frequency changes in stimulus waveforms above a 'pitch saturation limit' in the region of 400 Hz^{4,6–9}. Thus, many of the temporal details present in CA stimuli are not accessible to the typical user. In addition, the simultaneous presentation of stimuli may produce significant interactions among channels through vector summation of the electric fields from each electrode^{6,10,11}. The resulting degradation of channel independence would be expected to reduce the salience of channel-related cues.

The CIS strategy addresses the problem of channel interactions through the use of interleaved nonsimultaneous stimuli (Figs 1b and 2c). Trains of balanced biphasic pulses are delivered to each electrode with temporal offsets that eliminate any overlap across channels. The amplitudes of the pulses are derived from the envelopes of bandpass filter outputs. In contrast to the four-channel CA strategy, five or six bandpass filters (and channels of stimulation) generally are used in the CIS system to take advantage of additional implanted electrodes and reduced interactions among channels. The envelopes of the bandpass outputs are formed by rectification and lowpass

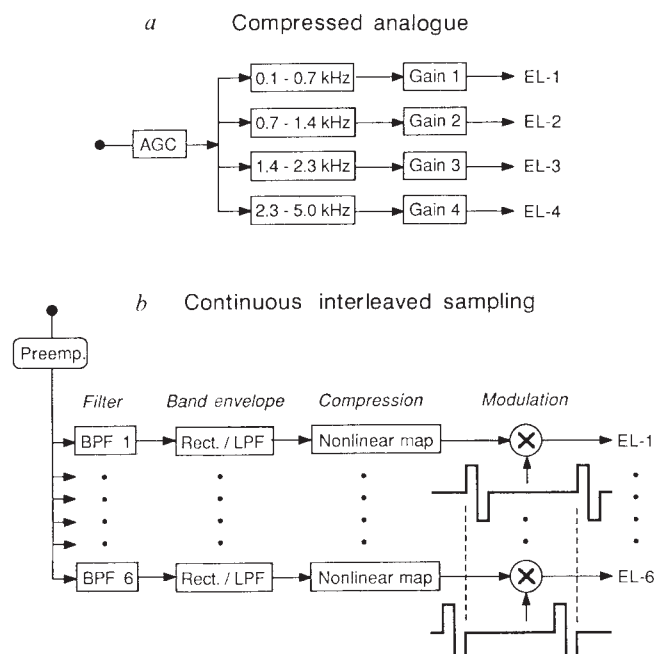


FIG. 1 Block diagrams of major processing steps in the CA and CIS strategies. *a*, The CA strategy uses a broadband AGC, followed by four channels of bandpass filtering (with the indicated frequencies) and adjustable gain controls. The outputs of the gain stages are connected to four intracochlear electrodes (EL-1 to EL-4). *b*, The CIS strategy uses a preemphasis filter (Preemp.) to attenuate strong low-frequency components in speech that otherwise might mask important high frequency components (high frequency emphasis is accomplished in the CA strategy by adjustment of the channel gain controls). The preemphasis filter is followed by five or six channels of processing. Each channel includes stages of bandpass filtering (BPF), envelope detection, compression, and modulation. The envelope detector consists of a rectifier (Rect.) followed by a lowpass filter (LPF). Carrier waveforms for two of the modulators are shown immediately below the two corresponding multiplier blocks.

TABLE 1 Individual and average scores from the tests of Fig. 3

Subject	Spondee		CID		SPIN		NU-6		Tracking	
	CA	CIS	CA	CIS	CA	CIS	CA	CIS	CA	CIS
SR2	92	96	100	100	78	96	56	80	81	94
SR3	52	96	66	98	14	92	34	58	51	89
SR4	68	76	93	95	28	70	34	40	—	—
SR5	100	100	97	100	94	100	70	80	—	—
SR6	72	92	73	99	36	74	30	49	43	56
SR7	80	100	99	100	66	98	38	71	51	68
SR8	68	100	80	100	36	94	38	66	56	94
Average	76	94	87	99	50	89	43	63	56	80

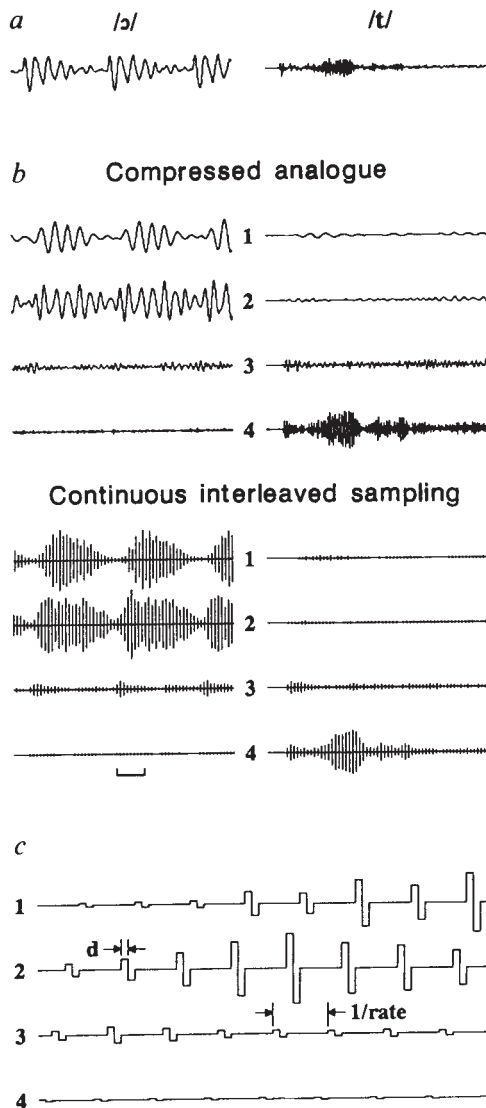


FIG. 2 Waveforms produced by simplified implementations of the CA and CIS strategies. *a*, Preemphasized (6 dB per octave attenuation below 1.2 kHz) speech inputs. Inputs corresponding to a voiced speech sound ('aw') and an unvoiced speech sound ('t') are shown in the left and right columns, respectively. The duration of each trace is 25.4 ms. *b*, Stimulus waveforms for the two processing strategies. The waveforms are numbered by channel, with channel 1 delivering its output to the apicalmost electrode. To facilitate comparisons between strategies, only four channels of CIS stimulation are illustrated here. In general, five or six channels have been used for that strategy. The pulse amplitudes reflect the envelope of the bandpass output for each channel. In actual implementations the range of pulse amplitudes is compressed using a logarithmic or power-law transformation of the envelope signal. *c*, Expanded display of CIS waveforms (from the bracketed interval in *b*). The pulse duration per phase ('d') and the period between pulses on each channel ('1/rate') are indicated. The sequence of stimulated channels is 4-3-2-1. The total duration of each trace is 3.3 ms.

filtering. Finally, the amplitude of each stimulus pulse is determined by a logarithmic or power-law transformation of the corresponding channel's envelope signal at that time. This transformation compresses the signal into the dynamic range appropriate for that channel.

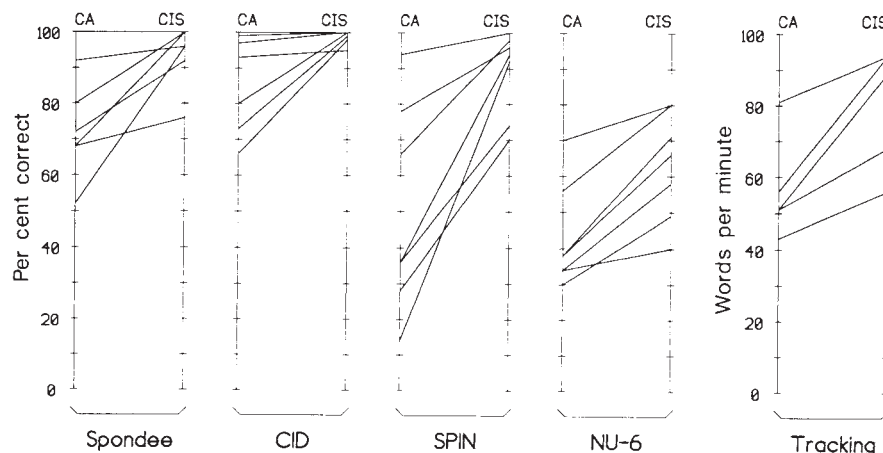
A key feature of the CIS strategy is its relatively high rate of stimulation on each channel. Other pulsatile strategies present sequences of interleaved pulses across electrodes at a rate equal to the estimated fundamental frequency during voiced speech and at a jittered or fixed higher rate during unvoiced speech^{12,13}. Rates of stimulation on any one channel rarely have exceeded 300 pulses per second (p.p.s.). In contrast, the CIS strategy generally uses brief pulses and minimal delays, so that rapid variations in speech can be tracked by pulse amplitude variations. The rate of stimulation on each channel usually exceeds 800 p.p.s. and is constant during both voiced and unvoiced intervals. The CIS strategy is designed to reduce channel interactions while still representing most or all of the temporal information that can be perceived by implant patients. The use of separate compression functions may further improve the temporal representation by exploiting the full dynamic range of each channel.

Comparisons of the CA and CIS strategies were made in tests with seven patients. All had excellent performance with their Ineraid clinical device^{1,2}, and were selected to be representative of the best patients using this or any other implant system¹³⁻¹⁷. The comparison tests included open-set recognition of words, sentences and paragraph material. Recognition of words and sentences was evaluated with four tests from the Minimal Auditory Capabilities (MAC) battery¹⁸, and recognition of paragraph material was evaluated using connected discourse tracking^{19,20}. The MAC tests included recognition of 50 one-syllable words from Northwestern University Auditory Test 6 (NU-6), 25 two-syllable words (spondees), 100 key words in the Central Institute for the Deaf (CID) sentences of everyday speech, and the final word in each of 50 sentences from the Speech Perception in Noise (SPIN) test (noise was not presented in the present study). All tests were conducted with hearing alone, and all tests except tracking used single presentations of recorded material with no feedback as to correct or incorrect responses. Each subject's own clinical device was used for the tests with the CA processor. Selection of parameters for the CIS processor was guided by preliminary tests of consonant identification^{5,12}. The standard four channels of stimulation were used for the clinical CA processors^{1,2}, whereas five or six channels were used for the CIS processors. All CIS processors had pulse durations of $\leq 102 \mu\text{s}$ per phase, pulse rates of ≥ 817 p.p.s., and a cutoff frequency for the lowpass filters of ≥ 400 Hz.

Results from the MAC and tracking tests are presented in Fig. 3 and Table 1. Both the high scores obtained with the CIS strategy and the substantial improvements made by each subject are impressive. Indeed, the sensitivity of several tests is limited for these subjects by ceiling (saturation) effects: five subjects scored $\geq 96\%$ for the spondee test using the CIS processor; all seven subjects scored $\geq 95\%$ for the CID test; and five subjects scored $\geq 92\%$ for the SPIN test. In addition, three of the tracking scores, of ≥ 89 words per min, approached those of normal

FIG. 3 Speech recognition scores for the CA and CIS processors. For every test, every subject scored higher, or repeated a score of 100% correct, using the CIS strategy. Paired *t* comparisons show that the increases across subjects are significant for the spondee ($P < 0.05$), SPIN ($P < 0.01$), NU-6 ($P < 0.002$) and tracking ($P < 0.02$) tests. In addition, a two-way analysis of the variance, using the five tests and two strategies as factors, demonstrates a highly significant difference between strategies ($F[1, 56] = 34.1$; $P < 0.0000005$), with no significant interaction between factors ($F[4, 56] = 1.4$; $P > 0.2$).

METHODS. In the MAC tests the subject's task was to recognize and report as many words as possible in the NU-6, spondee, CID, and SPIN lists. Because the tests with CA preceded those with CIS, we were concerned that practice or learning effects might favour the latter in comparisons of the two strategies. To evaluate this possibility, the CID and NU-6 tests were repeated with the CIS processor for five of the subjects, using a different recorded speaker and new lists of words and sentences. Practice or learning effects would be demonstrated by significant differences in the test/retest scores. But, no such differences were found ($P > 0.6$ for paired *t* comparisons of the CID scores; $P > 0.2$ for the NU-6 scores), and the scores from the first and second tests were averaged for all subsequent analyses. In the tracking test the subject's task was to repeat verbatim unfamiliar paragraphs read



by a trained speaker²⁰. When errors occurred, various techniques such as repeated presentation of phrases or words were used until verbatim repetition was achieved. The score was calculated by dividing the number of words in four paragraphs by the time required to complete those paragraphs. Because of schedule limitations, the tracking test was included for only five of the seven subjects.

hearing subjects¹⁹⁻²². Finally, the 80% score achieved by two of the subjects on the NU-6 test is in the range of scores obtained by people with mild-to-moderate hearing losses when taking the same test^{23,24}.

The improvements are even more striking considering the large disparity in experience with the two processors. At the time of our tests each subject had 2-5 years of daily experience with the CA processor, but only several hours of experience over a few days with CIS. In previous studies involving within-subject comparisons, such differences in experience have strongly favoured the processor with the greatest duration of use^{25,26}.

Together these results establish new levels of performance for cochlear implants. Factors contributing to this performance may include (1) reduction in channel interactions through the use of nonsimultaneous stimuli, (2) use of five or six channels instead of four, (3) representation of rapid envelope variations through the use of high pulse rates, (4) preservation of amplitude cues

with channel-by-channel compression, and (5) the shape of the compression function. Studies are in progress to evaluate possibilities 1-3 and 5. Preliminary results suggest that all of these factors affect performance, and that factors 1 and 3 are especially important.

In addition to their obvious implications for the treatment of deafness, the present results demonstrate that a simplified representation of speech is able to support relatively high levels of open-set recognition. The information presented by the CIS strategy is limited to envelope variations in five or six bands, with the maximum frequency of the variations (lowpass filter cutoff) typically set at 400 Hz. No specific features of the speech input, such as the fundamental or formant frequencies of voiced sounds, are extracted explicitly. The present results complement existing studies of simplified acoustic representations of speech for normal hearing listeners²⁷⁻³² and provide further insight into the minimal cues necessary for speech understanding. □

Received 10 May; accepted 3 June 1991.

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ACKNOWLEDGEMENTS. We thank M. Dorman, R. Tyler, M. Lowder and K. Dankowski for their help in identifying subjects and B. Moore, C. van den Honert and B. Weber for their editorial comments. We also thank each of the subjects for their time and interest. This work was supported by the NIH.

Formation of β -amyloid protein deposits in brains of transgenic mice

D. Quon, Y. Wang, R. Catalano, J. Marian Scardina, K. Murakami* & B. Cordell†

California Biotechnology Inc., 2450 Bayshore Parkway, Mountain View, California 94043, USA

* Present address: Research Institute, Daiichi Pharmaceutical Co., 16-13, Kita-kasi 1-Chome Edogawa-ku, Tokyo 134, Japan

† To whom correspondence should be addressed

DEPOSITS of β -amyloid are one of the main pathological characteristics of Alzheimer's disease. The β -amyloid peptide constituent (relative molecular mass 4,200) of the deposits is derived from the β -amyloid precursor protein (β -APP) which is expressed in several different isoforms¹⁻⁶. The two most prevalent β -APP isoforms are distinguished by either the presence (β -APP751) or absence (β -APP695) of a Kunitz serine protease inhibitor domain. Changes in the abundance of different β -APP messenger RNAs in brains of Alzheimer's disease victims have been widely reported⁷⁻¹². Although these results have been controversial, most evidence favours an increase in the mRNAs encoding protease inhibitor-containing isoforms of β -APP and it is proposed that this change contributes to β -amyloid formation⁹⁻¹². We have now produced an imbalance in the normal neuronal ratio of β -APP isoforms by preparing transgenic mice expressing additional β -APP751 under the control of a neural-specific promoter. The cortical and hippocampal brain regions of the transgenic mice display extracellular β -amyloid immunoreactive deposits varying in size (<5–50 μ m) and abundance. These results suggest that one mechanism of β -amyloid formation may involve a disruption of the normal ratio

of neuronal β -APP isoform expression and support a direct relationship between increased expression of Kunitz inhibitor-bearing β -APP isoforms and β -amyloid deposition.

A chimaeric gene was constructed between the human β -APP751 complementary DNA and the rat neural-specific enolase (NSE) promoter, termed NSE: β -APP751. The rat NSE promoter directs the neural-specific expression of β -galactosidase in transgenic mice¹³ and we have confirmed this using a NSE promoter fragment slightly truncated at the 5' terminus (our unpublished results). The promoter fragment containing the 5' untranslated region of NSE and a roughly 1.2-kilobase (kb) intron in this domain was fused to the β -APP751 cDNA such that the initiator methionine of NSE was replaced with the initiator methionine of β -APP751. Nine of 44 mice that developed from embryos injected with NSE: β -APP751 DNA carried the transgene. Three pedigrees were selected for extensive characterization: founders 10 (F10), 11 (F11) and 24 (F24). Homo- and hemizygotic states and transgene copy numbers were determined by comparison to the endogenous single copy β -APP mouse gene using Southern blot hybridization with a probe common to both mouse β -APP and human β -APP751 (Table 1).

RNA expression of the inherited transgenes in the three pedigrees was investigated. Total brain RNA was isolated both from positive and from wild-type control animals, reverse transcribed, and a specific DNA subfragment amplified by polymerase chain reaction (PCR). Primers for PCR were designed such that only transcripts derived from the transgene would be amplified, that is, one primer hybridizes to the NSE 5' untranslated region and the other to the 5' coding domain of β -APP. The NSE PCR primer corresponds to a site located upstream of the intron so that amplification of contaminating genomic DNA or unprocessed transcripts could be detected. A predicted 373-base pair (bp) fragment is amplified from reverse-transcribed RNA prepared from each transgenic animal but not from wild-type mice (Fig. 1a). As a control, half of the reverse-transcribed RNA was amplified with a primer for the native

isolated on the basis of the published sequence²² and the 2.3-kb fragment used for the chimaeric gene isolated by PCR. PCR primers were designed to generate *Bgl*II and *Nco*I sites at the 5' and 3' terminus of the fragment, respectively. A *Nru*I(position 123)–*Xmn*I(position 2,665) fragment was isolated from β -APP751 cDNA⁴, and was ligated with the 2.3-kb NSE fragment harboured in a derivative of pcDV1 plasmid containing the simian virus 40 (SV40) late region polyadenylation signal²³. A linear fragment of NSE: β -APP751 was prepared by cleavage with *Sal*I and *Nde*I and was injected into fertilized embryos of the J1 strain of mouse²⁴. The J1 strain, developed by Eric Bradford at the University of California Davis, was chosen for its large litter size. For genotype and copy number determinations, 40 μ g of tail DNA was digested with *Bgl*II and electrophoresed on 0.8% agarose gels. Southern blots were prepared²⁵ and hybridized with an oligonucleotide probe (5'-ATGGATGTGACTGTTTCTTCTTCA-3') radiolabelled with ³²P by T4 kinase. Blots were hybridized at 60 °C in 6 $\times$ SET (1 $\times$ SET = 0.15 M NaCl, 30 mM Tris-HCl pH 8.0, 2 mM EDTA) with 5 $\times$ Denhardt's solution and washed at 60 °C for 40 min in 6 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M Na-citrate). For transcriptional analyses, 2 μ g of total brain RNA was reverse transcribed with oligo(dT)₁₂₋₁₈, after which the reaction was divided into two equal aliquots. PCR²⁶ was done on one aliquot using NSE (5'-CACCGCCACCGCTGAGTCTGCAGTCTCG-3') and β -APP (5'-TCTTGCACTGCTTGCAGCCCGCTTGACC-3') primers and on the second aliquot with the same β -APP primer and a primer to the secretory signal sequence of β -APP (5'-TTGGCACTGCTCCTGCTGGCCGCTGGACG-3') in place of the NSE primer. DNA products were electrophoresed on 2% agarose gels, visualized by staining with ethidium bromide, then Southern blotted²⁵ and hybridized with a ³²P-labelled oligonucleotide probe to the NSE: β -APP751 fusion sequence (5'-AGATCCCCAGCACCGATGCTGCCCGGTTTG-3'). Blots were hybridized at 65 °C in 6 $\times$ SET and washed at 65 °C for 40 min in 4 $\times$ SSC. Protein homogenates were made from total brain¹⁴ and 50 μ g of each sample was electrophoresed on 7.5% SDS-polyacrylamide gels²⁷. A western blot²⁸ was developed using a 1:500 dilution of antiserum and ¹²⁵I-labelled protein A. The antiserum was raised against full-length human β -APP695 expressed by a recombinant vaccinia virus¹⁶. Identically prepared gels stained with Coomassie blue dye confirmed that equivalent amounts of protein were loaded for each sample.

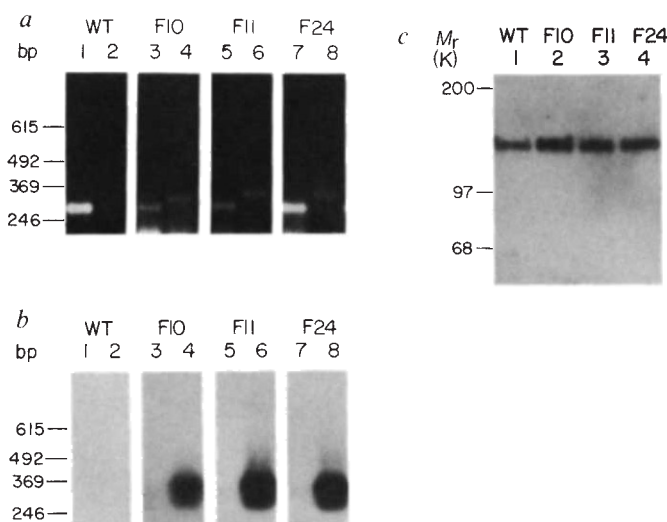


FIG. 1 NSE: β -APP751 expression in brain. *a*, Agarose gel electrophoresis of reverse transcribed PCR products visualized with ethidium bromide staining. Even numbered lanes, reverse transcribed PCR products from wild-type (WT), NSE: β -APP751 founder 10 (F10), founder 11 (F11) and founder 24 (F24) RNA primed with primers specific for NSE: β -APP751 RNA. Odd numbered lanes, reverse transcribed PCR products from RNA primed with primers specific for endogenous β -APP RNA. *b*, Southern blot of above reverse-transcribed PCR products using ³²P-labelled oligonucleotide probe specific for the NSE: β -APP751 chimaeric gene. Lanes are the same as in *a*. *c*, Western blot analysis of β -APP in brain. Lane 1, WT; 2, F10; 3, F11; 4, F24 total brain protein immunoblotted with β -APP antiserum.

METHODS. An ~8-kb rat genomic fragment containing the NSE gene was

β -APP secretory signal sequence and the same 5' coding domain β -APP primer to produce a 307-bp DNA fragment representing amplification from endogenous β -APP RNA (Fig. 1a). When all the PCR reaction products are hybridized with a probe bridging the junction between NSE and β -APP751 sequences, only the products derived from the transgenic brains hybridize, documenting the authenticity of the 373-bp PCR product (Fig. 1b).

Western blots were made to evaluate changes in protein expression in the brains of the transgenic animals. Equal amounts of total protein from whole brain homogenates were electrophoresed on polyacrylamide gels, transferred to a membrane then reacted with polyclonal serum raised against full-length β -APP. A band (or set of unresolved bands) of relative molecular mass of about 130,000 (130K), corresponding to the reported average size of mammalian brain β -APP isoforms¹⁴⁻¹⁶, is observed in the control, as well as in each transgenic protein homogenate (Fig. 1c). This signal is increased in the NSE: β -APP751 samples relative to the wild-type sample suggesting globally elevated β -APP751 expression in the transgenic brains. To obtain a more refined examination of NSE: β -APP751 expression and its effects, we used immunocytochemistry.

A panel of monoclonal antibodies was prepared using a synthetic peptide corresponding to residues 1-28 of the β -amyloid protein as the immunogen. The specificity of the monoclonals was established by immunoperoxidase staining of brain sections from Alzheimer's disease victims (Fig. 2a, b). Sections were prepared from brains of NSE: β -APP751 transgenic mice, as well as from wild-type mice and both were stained in parallel with one monoclonal, 4.1 (Table 1). Reproducibly greater immunoperoxidase reactivity is observed in neurons and as fine puncta throughout the neuropil of the transgenic brains compared with the immunoreactivity visualized in brains from wild-type mice. A pronounced staining of neuritic processes is also noticeable (Fig. 2d). This enhancement of arbor-forming neuronal processes is most evident in the stratum flanking the pyramidal cell layer of the CA-1 and CA-3 regions of transgenic

TABLE 1 Summary of mice used for immunohistology

Line	Animal	Sex	Age*	Genotype	Copy number†	Deposits‡
<u>NSE:β-APP751</u>						
10	0	F	12	Aa	1	+++
	31§	F	7	Aa		++
	168	F	5	Aa		+++
	334	M	2	AA		+
11	0	M	15	Aa	4	+++
	51	M	12	Aa		+
	236	M	4	AA		+++
	287	F	3	AA		+
24	77	M	8	Aa	8	+
	201	F	5	AA		+
<u>Wild type</u>						
	1	F	4	NA	NA	—
	2	F	4			—
	3	M	5			—
	4	M	3			—
	5	M	9			—
	6	F	12			—
	7	M	14			—

M and F indicate male and female mice, respectively; AA and Aa represent homozygotic and hemizygotic animals, respectively; NA, not applicable.

* Months.

† Haploid.

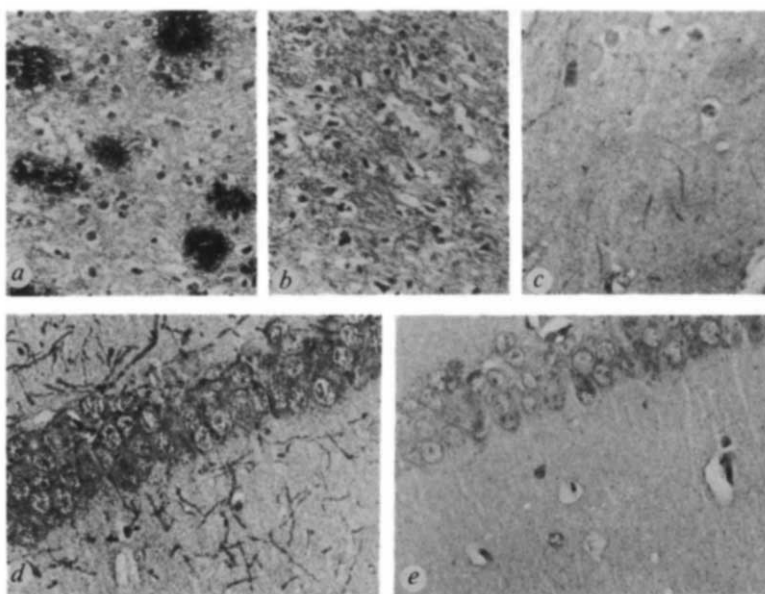
‡ $>5 \mu\text{m}$ in size, (—) none, (+/+ +/+++) relative abundance of deposits, that is, + indicates <5 ; ++ indicates 5-10; +++ indicates >10 deposits per section as an average of multiple sections stained.

§ NSE: β -APP751 F10, number 31 died of unknown cause.

hippocampi. Both neuronal and process staining are fully competed by earlier incubation of the antibody with the synthetic β -amyloid peptide. Neuritic staining in the transgenic brains is also detected using antibodies raised against full-length β -APP (Fig. 2c), indicating full-length β -APP is present in the neuritic

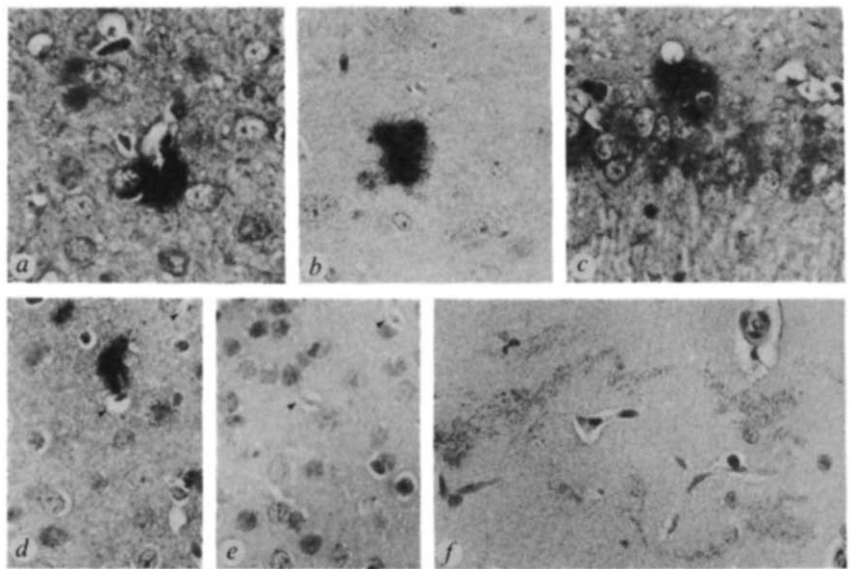
FIG. 2 Immunoperoxidase staining of human and mouse brain. Human Alzheimer's disease tissue section from caudal hippocampus stained with 4.1 antibody without (a) and an adjacent section (b) with preincubation with the β -amyloid synthetic peptide immunogen ($\times 250$). Hippocampal CA-1 field of NSE: β -APP751 F10 (number 334) stained with antibodies to full-length β -APP (c); pyramidal cell layer of CA-1 region from NSE: β -APP751 F10 (number 334) stained with 4.1 antibody (d); same region from wild-type mouse (number 3) identically stained with 4.1 antibody (e) ($\times 500$).

METHODS. A synthetic peptide corresponding to residues 1-28 of the β -amyloid protein^{1,2} was prepared and self-aggregated by freezing and thawing. The peptide aggregate was mixed with methylated bovine serum albumin and adjuvant for immunizing and boosting mice. Hybridomas from the sensitized spleen cells were generated²⁹. Clones secreting anti-peptide antibodies were expanded and subcloned by limiting dilution. The epitope recognized by each monoclonal was mapped to the N-terminal 10 residues of the β -amyloid protein. For analysis of mouse brains, brains were removed and fixed with 4% paraformaldehyde, embedded in paraffin and 6 μm coronal midbrain sections made. Sections were deparaffinized, rehydrated, treated for 30 min with 0.3% H_2O_2 , then with 80% formic acid for ~ 2 min. Sections were next incubated at 37°C for 30 min with a 1/20 dilution of conditioned medium from the hybridoma secreting the 4.1 antibody. An anti-mouse avidin-biotinylated horseradish peroxidase (ABC) kit was used according to supplier's recommendations (Vector, Burlingame, California) and the horseradish peroxidase visualized with 3,3'-diaminobenzidine. Staining for full-length β -APP used the antiserum used for western blotting in c at a 1:500 dilution and an anti-rabbit ABC kit. Formic acid treatment was omitted for β -APP staining. Sections were counterstained with haematoxylin and eosin. Human brain sections were from individuals clinically diagnosed with Alzheimer's disease. The human



sections were prepared and stained identically as mouse tissue sections except they were treated with 98% formic acid for 10 min. For competition experiments, the antibody diluent was preincubated at 4°C for 12 h then 37°C for 30 min with 250 $\mu\text{g ml}^{-1}$ 1-28 β -amyloid synthetic peptide before application. Congo red, thioflavin S and silver staining were done using published procedures^{18-21,30}.

FIG. 3 Immunoreactive deposits in NSE: β -APP751 brains. *a*, Compact deposit in frontoparietal cortex of F11 (number 0); *b*, compact deposit in thalamus of F11 (number 236); *c*, compact deposit in hippocampal CA-2 field of F11 (number 0); *d*, cluster of deposits in frontoparietal cortex of F10 (number 168); *e*, adjacent section as in *d* but antibody preincubated with β -amyloid synthetic peptide before staining (arrowheads demark same capillaries in field of *d* and *e*); *f*, amorphous deposits in the hippocampal stratum moleculare of F11 (number 236) ($\times 500$). Immunocytochemistry and competition were performed as described in the legend to Fig. 2.



processes, although the possibility that the neurites also contain β -amyloid protein cannot be excluded.

Extracellular immunoreactive deposits are also consistently seen in the brain sections from each of the three transgenic lines stained with the 4.1 monoclonal which are not seen in sections from wild-type animals identically stained. The immunoreactive deposits vary in size, shape and frequency. Examples of compact deposits 10–50 μm in diameter are shown in Fig. 3*a–d*. These immunoreactive deposits tend to occur in clusters and are most frequently observed in the cortex and hippocampus although occasionally they have been found in the thalamus and striatum. A second type of immunoreactive extracellular deposit is reproducibly seen in transgenic brain sections which is lacking in control brain sections. This type of deposit is diffuse, amorphous and granular (Fig. 3*f*). Detection of extracellular deposits in the tissue sections from the transgenic animals required treatment with formic acid. Immunoreactivity of these structures also could be competed by the β -amyloid peptide (Fig. 3*e*). In a preliminary survey, antibodies to full-length β -APP stained extracellular deposits of similar morphology as those stained by the 4.1 monoclonal. Also, in general, the deposits are stained by silver salts, infrequently by thioflavin S, but not by Congo red, corroborating a preamyloid-like composition (data not shown). Owing to the small group of animals analysed, it is difficult to make a correlation between the frequency of deposit appearance with age, genotype or sex (Table 1).

Although there may exist several different mechanisms promoting β -amyloid formation¹⁷, the observed increased level of Kunitz inhibitor-containing β -APP isoform RNA in neurons of Alzheimer's disease brains suggests that Kunitz inhibitor β -APP isoform overexpression may be one mechanism^{9–12}. The three NSE: β -APP751 transgenic lines which have moderately increased neuronal expression of β -APP751 and form extracellular β -amyloid immunoreactive deposits (as well as our preliminary data on NSE: β -APP695 transgenic lines which do not) support this hypothesis. The two types of extracellular deposits, diffuse and compact, seen in the transgenic mice resemble several β -amyloid structures typically seen in the brains of Alzheimer's disease victims, specifically preamyloid and preamyloid plaques^{18–21}. It will be of interest to determine whether the quality and/or quantity of deposits change in an age-dependent manner and if the mice display other pathological features characteristic of Alzheimer's disease. □

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ACKNOWLEDGEMENTS. We thank G. Anderson, S. Donahue and A. Moyer for performing transgenic embryology, A. Lam, U. Masharani, L. Carstensen and P. Hummel for their technical assistance and contributions in the development and care of transgenic animals, T. Finch for providing human brain sections, G. Murphy for viewing stained tissue sections, and E. Stoelting for artwork. This work was supported by Daiichi Pharmaceutical Co. of Tokyo, Japan.

Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores

Ludwig Missiaen*, Colin W. Taylor† & Michael J. Berridge*

* AFRC Laboratory of Molecular Signalling, Department of Zoology, University of Cambridge, Downing Street, Cambridge CB2 3EJ, UK
† Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK

Inositol 1,4,5-trisphosphate (InsP_3) functions as a second messenger to mobilize Ca^{2+} from intracellular reservoirs¹. The release mechanism displays all-or-none characteristics^{2,3}, that may account for other observations that the InsP_3 -induced mobilization of Ca^{2+} is quantal^{4–6}. Quantal release may depend on the sensitivity of the InsP_3 receptor being regulated by the Ca^{2+} concentration in the lumen of the endoplasmic reticulum⁷. We report here that

Received 31 January; accepted 25 June 1991.

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the InsP_3 -sensitive store in hepatocytes discharges spontaneously when overloaded with Ca^{2+} . The release, which is blocked by heparin, is preceded by an increasing sensitivity of the InsP_3 receptor to endogenous InsP_3 , and is promoted by those sulphhydryl reagents (oxidized glutathione and thimerosal) that induce Ca^{2+} oscillations in intact cells (ref. 8, and T. A. Rooney, D. C. Renard, E. J. Sass and A. P. Thomas, manuscript in preparation). This novel process could have a role in generating both Ca^{2+} oscillations and Ca^{2+} waves.

The spontaneous Ca^{2+} release from permeabilized hepatocytes (10^7 ml^{-1}) is illustrated in Fig. 1a. After the initial phase of Ca^{2+} sequestration to $103 \pm 5 \text{ nM}$, there is a pacemaker Ca^{2+} concentration ($[\text{Ca}^{2+}]$) rise culminating in a spontaneous spike with a peak $[\text{Ca}^{2+}]$ of $585 \pm 24 \text{ nM}$ ($n = 20$). The spike represents a synchronized Ca^{2+} release from InsP_3 -sensitive pools, because premature emptying of these pools with enough InsP_3 to keep them empty for the rest of the assay prevents its occurrence (Fig. 1b). Not all InsP_3 -sensitive Ca^{2+} pools contribute to the spike, because adding $10 \mu\text{M}$ InsP_3 on top of the $531 \pm 39 \text{ nM}$ $[\text{Ca}^{2+}]$ rise of the spike still increases the $[\text{Ca}^{2+}]$ by $629 \pm 55 \text{ nM}$, as compared with the $1,150 \pm 59 \text{ nM}$ $[\text{Ca}^{2+}]$ rise after an earlier addition ($n = 4$) (Fig. 1c).

Ca^{2+} release from overloaded mitochondria (Fig. 2a) does not contribute to the spontaneous spike, because $5 \mu\text{M}$ ruthenium red, which blocks this release⁹ (Fig. 2b), does not affect the amplitude of the spike. This finding also excludes the possibility that Ca^{2+} -induced Ca^{2+} release from the InsP_3 -insensitive Ca^{2+} pool contributes to the spike because ruthenium red also inhibits this release¹⁰. Furthermore, increasing the

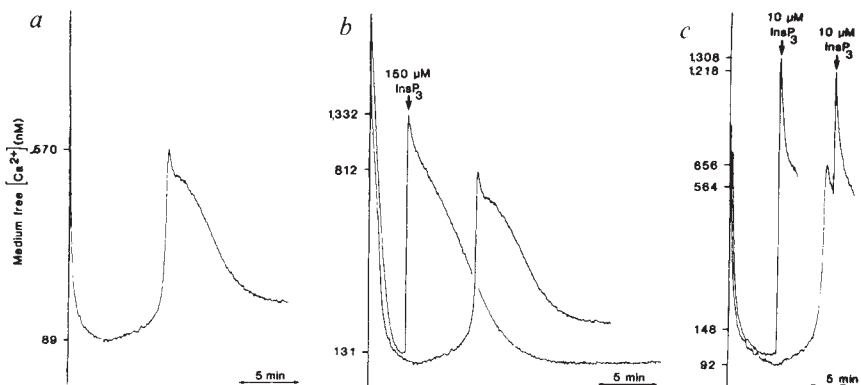
cytosolic $[\text{Ca}^{2+}]$ does not empty non-mitochondrial Ca^{2+} pools, as two separate additions of 1 nmol Ca^{2+} during the latency preceding the spike (Fig. 2c), as well as repeated 5-nmol Ca^{2+} pulses (Fig. 2d) do not trigger further release.

Diluting the cells to $5 \times 10^6 \text{ ml}^{-1}$ abolishes the spike, but this can be restored in a dose-dependent manner by the sulphhydryl reagent oxidized glutathione (GSSG) (Fig. 3a). As GSSG has no effect on the initial rate of Ca^{2+} sequestration, it does not inhibit the Ca^{2+} pumps while sensitizing the release mechanism responsible for the Ca^{2+} spike. Dithiothreitol (8 mM) blocks this effect. The sulphhydryl agent thimerosal ($50 \mu\text{M}$) also produces a spike, whereas $400 \mu\text{M}$ *N*-ethylmaleimide has no effect. The long latency before the $[\text{Ca}^{2+}]$ rises cannot be explained by the time needed to oxidize sulphhydryl groups, because 4 mM GSSG causes an immediate Ca^{2+} release when added after 17 min (Fig. 3b).

The Ca^{2+} response to 50 nM InsP_3 increases during the latency period (Fig. 3c), representing a sensitization of the InsP_3 receptor, because the release induced by 50 nM relative to that by $10 \mu\text{M}$ InsP_3 increases from 30% at the beginning of the latency to 60% immediately before the spike. Adding 5 nM InsP_3 immediately before the spike produces the same rise in $[\text{Ca}^{2+}]$ as 50 nM given at the beginning of the latency. The spontaneous spike seems to empty those Ca^{2+} pools which became most sensitive to InsP_3 , because 50 nM InsP_3 is unable to trigger a significant Ca^{2+} release after the spike (Fig. 3c). No such sensitization occurs in the absence of GSSG, where no spike develops (Fig. 3d). Heparin ($50 \mu\text{g ml}^{-1}$), an antagonist of the InsP_3 receptor¹¹, completely prevents both the effect of adding

FIG. 1 Spontaneous Ca^{2+} release from InsP_3 -sensitive Ca^{2+} stores in permeabilized hepatocytes (10^7 ml^{-1}). a, Pattern of Ca^{2+} sequestration in the absence of mitochondrial inhibitors; b, InsP_3 ($150 \mu\text{M}$) prevents the spike; c, InsP_3 ($10 \mu\text{M}$) added on top of the spike, releases less Ca^{2+} than before the spike.

METHODS. Rat hepatocytes, prepared by collagenase digestion, were kept in cold NaHCO_3 -buffered Eagle's medium containing 2% (w/v) BSA until required⁵. Cells were washed twice and finally resuspended in a medium containing 120 mM KCl, 30 mM HEPES (pH 7.3), 1 mM MgCl_2 , 1 mM ATP, 25 mM phosphocreatine, 25 units ml^{-1} creatine phosphokinase and $5 \mu\text{M}$ fluo-3. Cells ($300 \mu\text{l}$) were transferred to a stirred cuvette at 37°C in a Perkin-Elmer LS5 luminescence spectrometer and Ca^{2+} uptake started by adding $50 \mu\text{M}$ digitonin. Traces represent emitted fluorescence at 530 nm (excitation at 503 nm) with the corresponding free $[\text{Ca}^{2+}]$ ($K_d = 864 \text{ nM}$), as a



function of time after permeabilization.

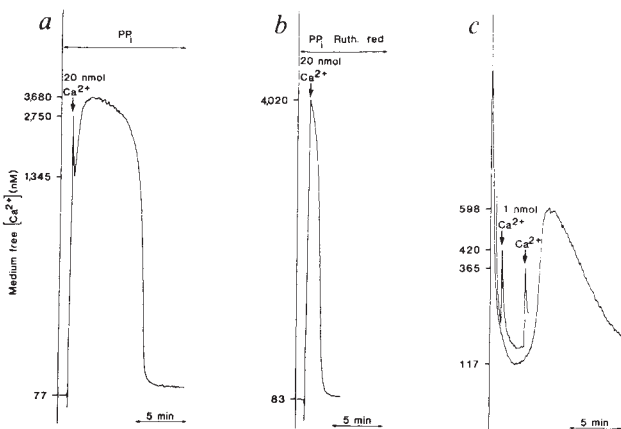
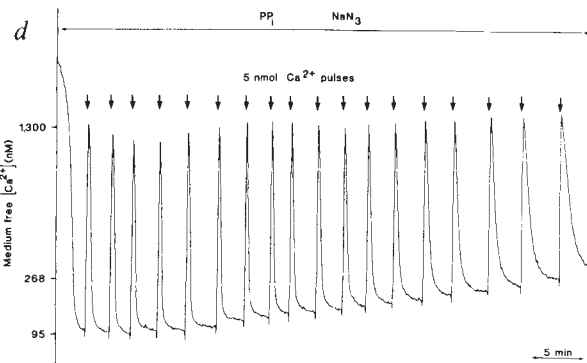


FIG. 2 Handling of Ca^{2+} pulses by permeabilized hepatocytes (10^7 ml^{-1}). a, A 20-nmol Ca^{2+} pulse triggers a subsequent Ca^{2+} release in an assay medium supplemented with 5 mM pyrophosphate (PP_i), which promotes Ca^{2+} release from mitochondria⁹; b, Prevention of this Ca^{2+} release by $5 \mu\text{M}$



ruthenium red. c, Effect of adding two pulses of 1 nmol Ca^{2+} ; d, Ca^{2+} pulses of 5 nmol each are added to cells resuspended in a medium supplemented with 10 mM NaN_3 as mitochondrial inhibitor and 5 mM pyrophosphate and washed once to remove cytosolic pyrophosphatase.

50 nM InsP_3 and the spontaneous spike, but does not affect the slow pacemaker rise (Fig. 3e). When added at the peak of the spike, heparin immediately curtails the response (Fig. 3f). Inhibition of InsP_3 binding by increasing the ATP concentration to 10 mM¹², or by adding 5 mM 2,3-bisphosphoglycerate¹², also prevents the spike. The spike therefore depends on the activation by the basal level of InsP_3 , which is 59 ± 4 nM ($n = 3$) (measured with an InsP_3 binding protein kit, Amersham). Dithiothreitol and heparin similarly suppress the spike, but not the pacemaker $[\text{Ca}^{2+}]$ rise, in concentrated cell suspensions, where a similar sensitization to InsP_3 occurs during the latency.

The mechanism responsible for this sensitization to InsP_3 appears to be a gradual overloading of the non-mitochondrial pools by Ca^{2+} leaking from the mitochondria. The mitochondrial Ca^{2+} content, assessed by measuring how much Ca^{2+} is released by 10 μM FCCP (trifluoromethoxycarbonyl cyanide phenylhydrazone), progressively decreases with time (Fig. 4a), probably because mitochondria leak Ca^{2+} when the $[\text{Ca}^{2+}]$ falls below their set point¹³. GSSG (4 mM) has no effect on this Ca^{2+} leak. The overloading of the pools and the spontaneous release of their Ca^{2+} are related, because adding extra Ca^{2+} , which is assumed to overload more rapidly the non-mitochondrial Ca^{2+} pools, triggers an earlier spike, without affecting its amplitude (Fig. 4b). Conversely, increasing the Ca^{2+} -accumulating capacity of the pools with precipitating anions abolishes the spike.

The spontaneous spike we have recorded from permeabilized hepatocytes could be related to the repetitive transients

described in intact hepatocytes (refs 14–16, and T. A. Rooney, D. C. Renard, E. J. Sass and A. P. Thomas, manuscript in preparation) and other cells^{8,17–20}. Ca^{2+} spikes in intact cells also originate from an InsP_3 -sensitive pool^{18,19} and their amplitudes are also half those after maximal agonist stimulation^{14,17}. The reduced latency after adding more Ca^{2+} (Fig. 4b) fits with the increased oscillation frequency in intact cells when Ca^{2+} influx is enhanced by increasing the dose of agonist^{14,16,17} or the external $[\text{Ca}^{2+}]$ ^{16,19,21–24}. In intact hepatocytes, classical Ca^{2+} transients, resembling those induced by agonists, are observed in response to *tert*-butyl hydroperoxide, which acts by oxidizing glutathione to GSSG (T. A. Rooney, D. C. Renard, E. J. Sass and A. P. Thomas, manuscript in preparation). Similarly, the sulphhydryl reagent thimerosal, but not *N*-ethylmaleimide, triggers a typical Ca^{2+} oscillation in hamster eggs⁸. On the basis of our observation on permeabilized hepatocytes, we propose that these sulphhydryl reagents induce oscillations by sensitizing the InsP_3 receptor to the endogenous level of InsP_3 .

The finding that the InsP_3 -sensitive store can spontaneously release its Ca^{2+} is consistent with the 'two pool' oscillator model¹, which emphasizes the importance of pool refilling as a prerequisite for spike initiation. But the original model has to be modified by assuming that a proportion of the InsP_3 -sensitive stores are functionally insensitive to InsP_3 by virtue of their location in the cell. At the low agonist doses that generate oscillations, the rise in InsP_3 concentration ($[\text{InsP}_3]$) is probably restricted to a very small region immediately below the plasma membrane²⁵. Emptying of the superficial InsP_3 -sensitive pools and the resulting enhanced Ca^{2+} entry will then charge up the InsP_3 -sensitive Ca^{2+} pools deeper in the cell that do not detect the rise in $[\text{InsP}_3]$. These pools then progressively sensitize to the ambient low level of InsP_3 until a critical Ca^{2+} content is reached and a Ca^{2+} spike triggered.

The main conclusion to emerge from our study is that the InsP_3 -sensitive store releases its Ca^{2+} spontaneously when overloaded with Ca^{2+} through a mechanism that requires a threshold level of InsP_3 and can be sensitized by sulphhydryl reagents such as GSSG and thimerosal. Calcium seems to induce its own release in two ways, not only by sensitizing the receptor to InsP_3 but also, once release begins, by exerting a positive feedback

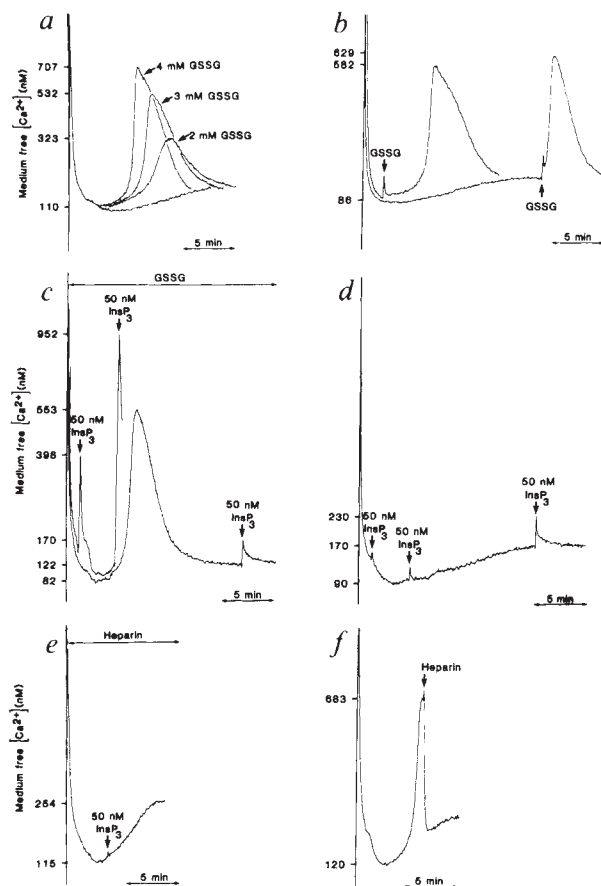


FIG. 3 Induction of a Ca^{2+} spike by GSSG in diluted cells ($5 \times 10^6 \text{ ml}^{-1}$) is the result of a sensitization to basal InsP_3 levels. *a*, Adding the indicated concentration of GSSG immediately before permeabilization induces a spike. *b*, Effect of later additions of 4 mM GSSG. *c*, *d*, Ca^{2+} release by three additions of 50 nM InsP_3 in the presence (*c*) and in the absence (*d*) of 4 mM GSSG. InsP_3 (50 nM) and heparin ($50 \mu\text{g ml}^{-1}$) are added as indicated in *e* and *f* in the presence of 4 mM GSSG.

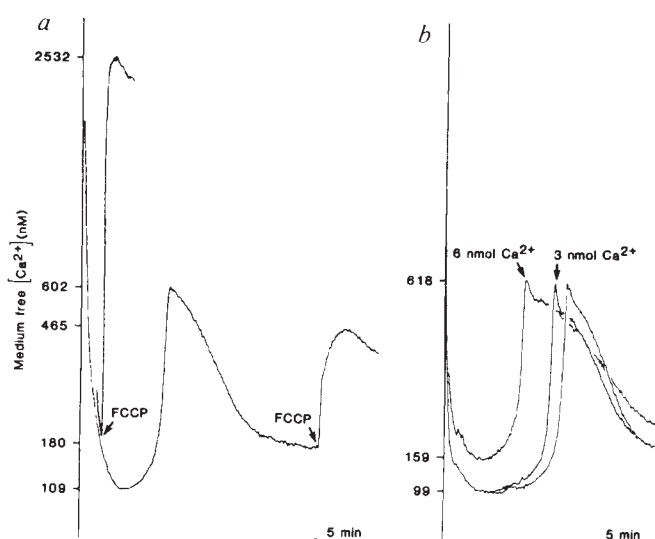


FIG. 4 The Ca^{2+} spike is the result of a gradual overloading of the non-mitochondrial Ca^{2+} pools. *a*, Decreasing Ca^{2+} release by 10 μM FCCP illustrates that the mitochondria lose Ca^{2+} , and thereby charge up the non-mitochondrial Ca^{2+} pools. *b*, Loading of the latter pools is enhanced by adding the indicated amount of Ca^{2+} immediately before permeabilization. Cell density, 10^7 ml^{-1} .

effect by promoting the Ca^{2+} -mobilizing action of InsP_3 (refs 26–28). In many respects, this spontaneous Ca^{2+} release closely resembles the agonist-dependent Ca^{2+} spiking behaviour of intact cells. □

Received 9 April; accepted 11 June 1991.

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ACKNOWLEDGEMENTS. InsP_3 was a gift from R. Irvine. L. M. is senior research assistant of the NFWO (Belgium). This work was supported by the Royal Society and EMBO (L.M.), the Wellcome Trust (C.W.T.) and Otsuka Pharmaceutical Company (M.J.B.).

ATP-sensitive K^+ channel in the mitochondrial inner membrane

Isao Inoue, Hideki Nagase*, Kuki Kishi
& Tomihiko Higuti*

Institute for Enzyme Research, and *Faculty of Pharmaceutical Sciences,
Tokushima University, Tokushima 770, Japan

MITOCHONDRIA take up and extrude various inorganic and organic ions, as well as larger substances such as proteins^{1–4}. The technique of patch clamping should provide real-time information on such transport and on energy transduction in oxidative phosphorylation. It has been applied to detect microscopic currents from mitochondrial membranes and conductances of ion channels in the 5–1,000 pS range in the outer and inner membranes^{5–10}. These pores are not, however, selective for particular ions. Here we use fused giant mitoplasts prepared from rat liver mitochondria to identify a small conductance channel highly selective for K^+ in the inner mitochondrial membrane. This channel can be reversibly inactivated by ATP applied to the matrix side under inside-out patch configuration; it is also inhibited by 4-aminopyridine and by glybenclamide. The slope conductance of the unitary currents measured at negative membrane potentials was 9.7 ± 1.0 pS (mean $\pm$ s.d., $n=6$) when the pipette solution contained 100 mM K^+ and the bathing solution 33.3 mM K^+ . Our results indicate that mitochondria depolarize by generating a K^+ conductance when ATP in the matrix is deficient.

The outer membranes of isolated rat liver mitochondria were removed by digitonin treatment to generate mitoplasts with their inner membrane matrix intact^{11,12}. These mitoplasts were then made spherical by hypotonic swelling^{11,12} and fused in a low-pH solution (pH 6.5) containing Ca^{2+} (ref. 13); fusion was stopped by changing to a low- Ca^{2+} (10^{-7} M) buffered medium (pH 7.2). Figure 1 shows these fused giant mitoplasts adhering tightly to

a coverslip. A cell attached patch clamp was achieved in a giant mitoplast of diameter 12 μm .

We recorded several types of single channel currents from the mitoplast membrane, including small amplitude currents that could be inhibited by ATP applied to the bath (matrix side) under inside-out configuration. Such ATP-sensitive currents were detected in 31 patches out of 94 inside-out patches. In 13 patches the currents at negative membrane potentials were not accompanied by other large amplitude currents.

The ATP-sensitive currents ran down gradually after making inside-out patches. As one membrane patch contained several ATP-sensitive channels, we could repeatedly demonstrate channel inactivation and reactivation by addition and removal of ATP to and from the bathing solution during the run-down period (~ 1 h). Figure 2a shows current traces at $V_m = -70$ mV at ~ 40 min after the inside-out patch was made, as only a few ATP-sensitive channels were still active, these records show clear single-channel events. The current consisted of a unit of -0.85 pA (Fig. 2b), but when two channels were active in the patch, the one open state was unstable and behaved like a substate of the dual open states. Such cooperative transitions between dual or multi states were observed when more channels were active in a patch, as can be seen from the current traces in Fig. 3b. These indicate that the ATP-sensitive channels may form a cluster and interact with each other. Figure 2c plots the open probability of the ATP-sensitive channel at various ATP concentrations in the bath relative to that before ATP application, and shows that the channel activity is half inhibited at ~ 0.8 mM, and almost blocked at >2 mM. By contrast, neither ADP nor GTP at 2 mM has any significant effect on channel

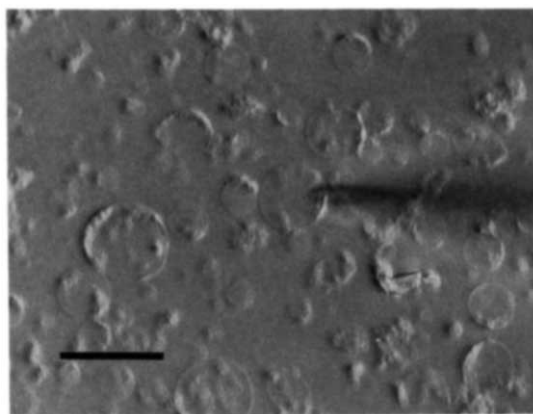


FIG. 1 Light micrograph of fused giant mitoplasts under Nomarski optics. Bathing solution, 33.3 mM KCl with 66.7 mM NaCl; temperature, 35 °C. A patch pipette was introduced onto a giant mitoplast of 12 μm diameter. Scale bar represents 20 μm .

METHODS. Mitochondria were isolated from fresh liver of male Wistar rats (~ 250 g) as described in ref. 21. They were then suspended in H medium (220 mM D-mannitol, 70 mM sucrose, 2.5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES)-NaOH buffer, pH 7.4, 0.5 mg ml⁻¹ BSA) and treated (100 mg protein per ml) with 12 mg ml⁻¹ purified digitonin for 20 min while stirring on ice. This mixture was diluted with 3 volumes of 7.5 $\times$ diluted H medium and washed twice in the same diluted medium by centrifuging at 10,000g for 10 min. Electron microscopy has shown that such mitoplasts have almost no outer membrane^{11,12}; we confirmed the absence of any monoamine oxidase activity, which is an enzyme located in the outer membrane²³. The mitoplasts were suspended in a low-pH medium (pH 6.5) to 0.4 mg protein per ml and mixed with an equal volume of a solution containing 20 mM CaCl_2 and 5 mM HEPES-KOH buffer, pH 6.5; the suspension was then aliquoted (4 ml) into plastic dishes (Falcon 1006, New Jersey) containing 3 or 4 coverslips and incubated for 30 min at 37 °C. Fusion was stopped by switching to a low- Ca^{2+} bathing solution containing 100 mM KCl, 7.5 mM Na-MOPS buffer, pH 7.2, 1 mM and 0.55 mM CaCl_2 . Fused giant mitoplasts spontaneously adhering to the coverslips were used in further experiments.

activity (data not shown). Mg^{2+} is not required for ATP blocking of the channel. Two current traces shown in Fig. 3 demonstrate that the channel activity partially inhibited by 1 mM ATP is further blocked by 5 mM 4-aminopyridine (a K^+ -channel blocker¹⁴) and by 5 μ M glibenclamide (which blocks ATP-sensitive K^+ channels¹⁵).

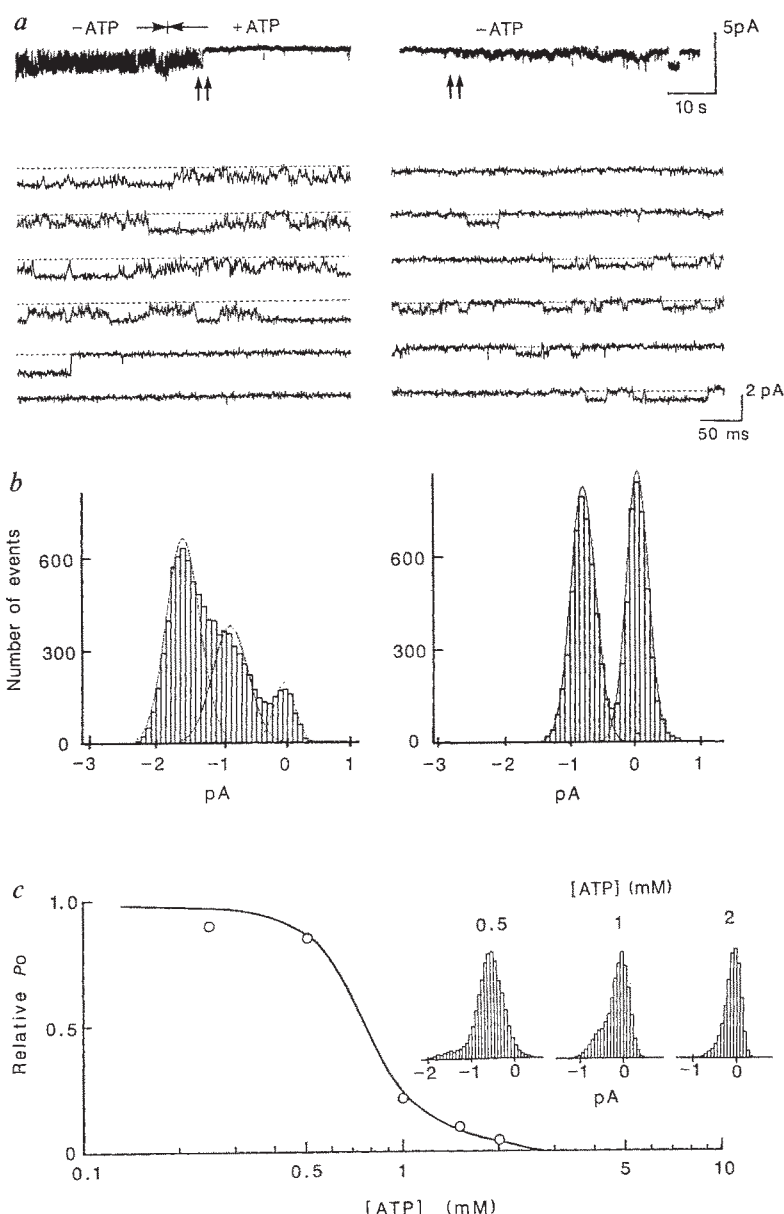
Figure 4 (left) shows ATP-sensitive currents at different membrane potentials (Fig. 4a), and large currents that appeared only at positive membrane potentials (Fig. 4b), recorded from the same inside-out patch with a pipette solution containing 100 mM K^+ and with a bath solution of 33.3 mM K^+ and 66.7 mM Na^+ . The $I-V$ relation for the ATP-sensitive currents (open circles in Fig. 4c) shows that current amplitude increases linearly with hyperpolarization in the negative voltage region and that reversal potential estimated by linear extrapolation to

the V_m axis is +27 mV, which is nearly equal to the equilibrium potential for K^+ (E_K) of +29.5 mV. The average value of the slope conductance from six experiments using the same ionic composition was 9.7 pS, s.d. ± 1.0 pS. The other voltage-dependent channel had a slope conductance of 105 pS, and a reversal potential of 0 mV (Fig. 4c, filled circles). The amplitude histograms in Fig. 4d indicate that the activity of the ATP-sensitive channel is weakly dependent on membrane potential, at least in the negative voltage region. The histogram at +70 mV is evidence for the two ionic channels. The ratio of the open probability of the two channels was, however, different in different patches.

At least three other types of ATP-insensitive larger conductance channels (~ 55 , ~ 100 , ~ 200 pS) were commonly observed apart from the ATP-sensitive channel. All these ATP-insensitive

FIG. 2 a, Current traces at $V_m = -70$ mV under inside-out patch mode demonstrating the reversible suppression effect of ATP on the channel activity of the mitochondrial inner membrane. When ATP (2 mM) was applied to the 33.3 mM K^+ and 66.7 mM Na^+ bathing solution and the pipette contained 100 mM K^+ , the ATP blockade appeared. Lower traces show the currents in the upper traces (between the two arrows) at a higher sweep velocity; dashed lines indicate zero current level. b, Amplitude histograms of the same current records before application of ATP (left), and after reactivation of the channels by removal of ATP (right). The channel of -0.85 pA has a slope conductance of 9.0 pS which was calculated from the current-voltage relation. c, Relative open probability (P_o) as a function of the ATP concentration in the bath. The current data were obtained from an inside-out patch at $V_m = -50$ mV. Amplitude histograms at three ATP concentrations are presented. The mean current amplitude was 0.72 pA, which corresponds to 9.5 pS when the reversal potential is assumed to be +26 mV (see Fig. 4). The patch was exposed to each testing solution with a different concentration of ATP for 40 s, and data from the last 20 s were collected for the amplitude histogram. All recordings were made in less than 5 min, which was a short enough time by comparison with the rate of spontaneous run-down of channel activity. The dissociation constant at the half-maximum inhibition is 8×10^{-4} M.

METHODS. Patch clamp was achieved onto fused giant mitoplasts of 7–20 μ m diameter in a 33.3 mM K^+ and 66.7 mM Na^+ solution containing 33.3 mM KCl, 66.7 mM NaCl, 7.7 mM Na-MOPS, pH 7.2, 2 mM EGTA, using a patch clamp amplifier (List L/M EPC7, Darmstadt, Germany). Patch pipettes were pulled from 1.5-mm-diameter borosilicate microhaematocrit tubing (Modulohm, Denmark), and the tip slightly heat-polished. Electrodes were filled with a 100 mM K^+ solution containing 100 mM KCl, 7.5 mM sodium-MOPS, pH 7.2, 1 mM EGTA, 0.55 mM $CaCl_2$. The portion near the tip was then silicone-coated with a drop of N,N -dimethyl-trimethylsilylamine (Fluka). Electrode resistance was 3–7 M Ω . Cell-attached patches were made using a small negative pressure (~ 0.5 cm H_2O) relative to atmospheric; 20–30 min was necessary to obtain a giga-seal patch. Seal resistance was 5–20 G Ω . Excised inside-out patches could be obtained simply by separating the electrode tip from the mitoplast monitoring the outwardly rectifying currents (see text) when detected. The bathing solution was perfused after making an excised patch. The electrode tip was placed near the inlet of perfusion fluid to minimize the delay between changing the solution and its reaching the membrane patch. This time lag was usually 10–20 s and could be due to diffusion inside the pipette where an excised membrane patch was formed. Because of this definite lag period, drug inhibition of channel activity could be separated from spontaneous run-down of channel activity. ATP (Sigma) was neutralized with 0.1 N KOH to make a 50 mM ATP stock which was aliquoted (500 μ l) in plastic tubes and stored at -20° C for up to 1 week. ATP was added to the testing solution when necessary. The membrane potential (V_m) under inside-out patch mode represents the electric potential of the bath (matrix side) referred to that of the pipette solution. Transmembrane currents from the pipette to the



bath are negative and are shown as downward deflections. Single channel currents were analysed with an IBM/AT-compatible computer (Supercom 386T) using the pCLAMP programme (Axon Instruments) after data acquisition with a 12-bit A/D converter (Labmaster DMA, Scientific Solutions) at a sampling frequency of 10 or 20 kHz through a 4-pole Bessel low-pass filter (1–3 kHz). All experiments were carried out at $35 \pm 2^\circ$ C.

channels had a reversal potential of ~ 0 mV when ionic compositions between the pipette and bathing solutions were different. One with a conductance of ~ 100 pS was very dependent on voltage (Fig. 4), showing fast open-close kinetics at positive membrane potentials. This channel was detected in 32 patches out of 94, and was present in 11 of 13 patches studied more extensively. It may be similar to the slightly anion-selective channel in mitoplasts from giant mitochondria in cuprizone-fed⁵ or untreated mice⁶. The two other channels were less voltage-dependent. The larger conductance channel was observed in 61 patches out of the 94, and the smaller conductance one in 22 patches.

This is, to our knowledge, the first demonstration of a highly ion-selective channel in the mitochondrial inner membrane.

From its electrophysiological and pharmacological properties we conclude that this channel is one of a family of ATP-sensitive K^+ channels (K_{ATP} channels) found in some cell membranes¹⁶. Our results suggest that mitochondria generate a K^+ conductance if the ATP level on the matrix side becomes deficient. This may lead to a depolarization of the inner membrane towards E_K , and would result in a change in water content of the mitochondria. It is not known how the K_{ATP} channel affects the physiological function of mitochondria: the channel activity in the inner membrane would dissipate the membrane potential of energized mitochondria, estimated to be in the region of -150 to -160 mV (ref. 17). It is known that mitochondria in living cells swell and contract by changing their water content¹⁸⁻²⁰, some of these changes being related to the respiratory chain:

FIG. 3 *a*, Current trace demonstrating that channel activity decreased by 1 mM ATP is further inhibited by 5 mM 4-aminopyridine (4AP) (Sigma); $V_m = -40$ mV. *b*, Trace showing that channel activity reduced by 1 mM ATP is suppressed by 5 μ M glybenclamide (Sigma); $V_m = -50$ mV. Lower traces show these currents at a higher sweep velocity after addition of ATP (left) and glybenclamide (right). Transitions marked by arrows indicate unitary currents having an amplitude of ~ 0.75 pA, which corresponds to 9.9 pS (assuming a reversal potential of $+26$ mV). Amplitudes of other current transitions were multiple(s) of the unitary value, suggesting that similar channels interact with each other.

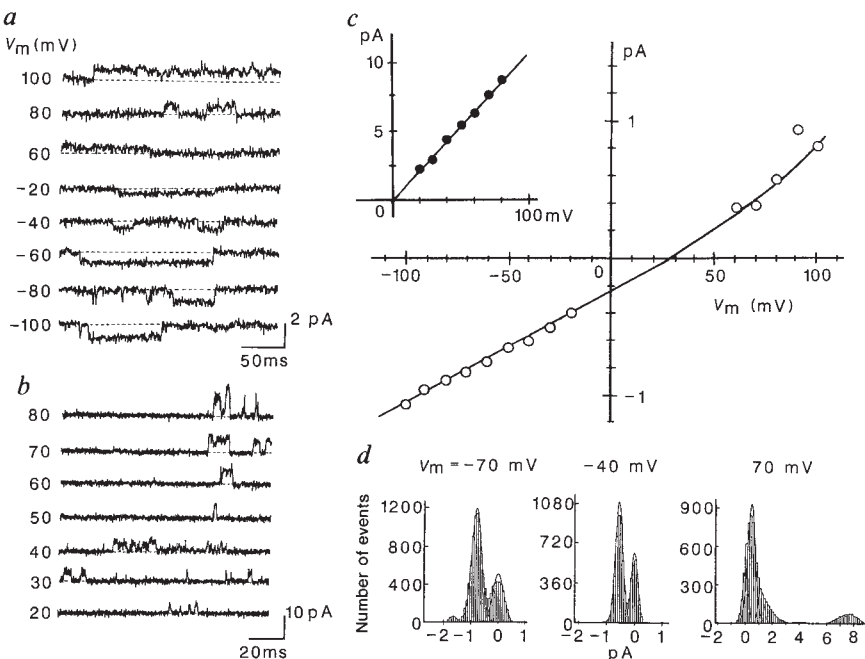
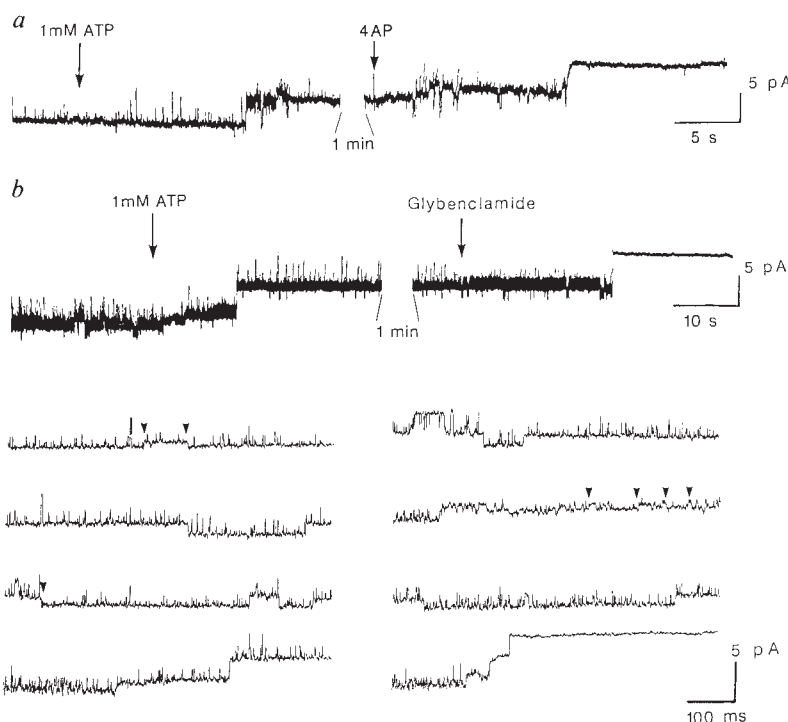


FIG. 4 *a*, ATP-sensitive single channel currents at various membrane potentials. Recordings were taken during 30–35 min after the inside-out patch was made. The pipette solution contained 100 mM K^+ , and the bathing solution 33.3 mM K^+ and 66.7 mM Na^+ . *b*, ATP-insensitive currents with a larger amplitude, which were recorded only at the positive membrane potentials in the same membrane patch. *c*, Current-voltage relations of the ATP-sensitive channel (open circles) and the ATP-insensitive channel (filled circles). Each value of the current amplitude was determined from a gaussian fit to the amplitude histogram. *d*, Amplitude histograms at $V_m = -70$ mV (left), at $V_m = -40$ mV (centre), and at $V_m = +70$ mV (right). At negative membrane potentials only the ATP-sensitive channel was active. P_o at $V_m = -70$ mV was 0.67, and was 0.65 at $V_m = -40$ mV. Two major peaks at $V_m = +70$ mV, one at 0.41 pA and the other at 7.55 pA, represent the ATP-sensitive and the voltage-dependent channels, respectively. Values of P_o of the ATP-sensitive and the voltage-dependent channels were 0.58 and 0.11, respectively. Current noise level increased at positive membrane potentials, which resulted in poor resolution of the current components in the amplitude histograms.

low water content is maintained during respiration under phosphorylating conditions, and mitochondria swell when adenine nucleotides or substrates are absent¹⁸. It remains to be determined whether the K_{ATP} channel is involved in the swelling-contraction cycle as an ATP sensor. □

Received 13 February; accepted 7 June 1991.

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ACKNOWLEDGEMENTS. We thank Q. Bone for discussion.

Independent inactivation of MPF and cytosstatic factor (Mos) upon fertilization of *Xenopus* eggs

Nobumoto Watanabe*, Tim Hunt†, Yoji Ikawa*‡ & Noriyuki Sagata§||

* Tsukuba Life Science Centre, The Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305, Japan

† ICRF Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK

‡ Tokyo Medical and Dental University, Yushima, Bunkyo, Tokyo 113, Japan

§ Division of Molecular Genetics, Institute of Life Science, Kurume University, 2432-3 Aikawa, Kurume, Fukuoka 830, Japan

IN vertebrates, mature eggs are arrested at the second meiotic metaphase by the cytosstatic factor (CSF)¹, now known to be the *c-mos* proto-oncogene product (Mos)^{2,3}. Fertilization or egg activation triggers a transient increase in the cytoplasmic free calcium^{4,5} and releases the meiotic arrest by inactivating maturation/mitosis-promoting factor (MPF)^{6,7}. CSF or Mos, which is also inactivated by the calcium transient^{8,9}, seems to stabilize MPF in mature eggs and CSF-injected embryos^{2,6,10}. Thus, it was assumed that CSF inactivation is the primary cause of MPF inactivation on meiotic release^{2,6,8,10–14}. We have directly compared the degradation kinetics of CSF (Mos) and MPF during meiotic release, using the same batch of *Xenopus* eggs. We report here that, at the molecular level, cyclin subunits of MPF are degraded before Mos is degraded and, at the physiological level, that MPF activity is inactivated before CSF activity during activation of *Xenopus* eggs. These results, in conjunction with circumstantial evidence, support the novel view that a calcium transient on fertilization induces a CSF-independent pathway for MPF inactivation, whereas CSF inactivation during meiotic release serves only to allow the fertilized egg to enter mitosis.

The degradation of cyclins B1 and B2, components of *Xenopus* MPF (ref. 15), is equivalent to the inactivation of MPF on

fertilization or egg activation¹¹. Hence, we first compared the degradation kinetics of cyclins and Mos on activation of ³⁵S-methionine-labelled mature eggs. Immunoprecipitation analysis showed that cyclin B1 degradation had started as early as 5 min after activation and was nearly complete 10 min after activation (Fig. 1a). Cyclin B2 was degraded slightly more slowly than

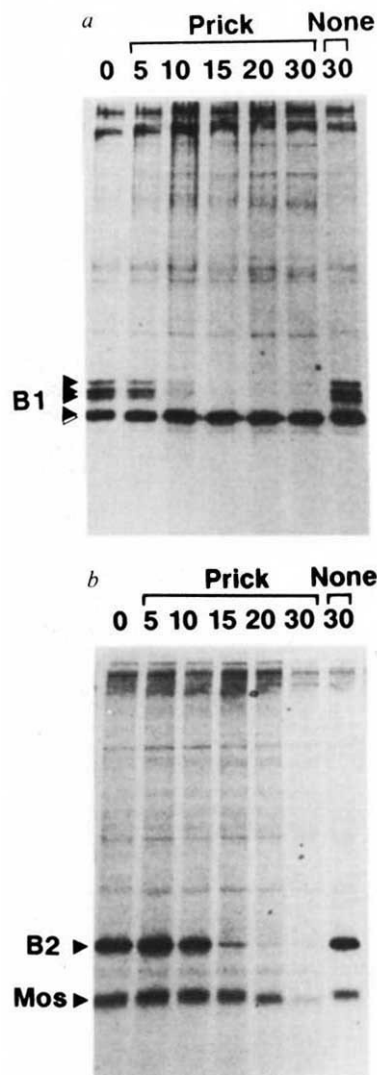
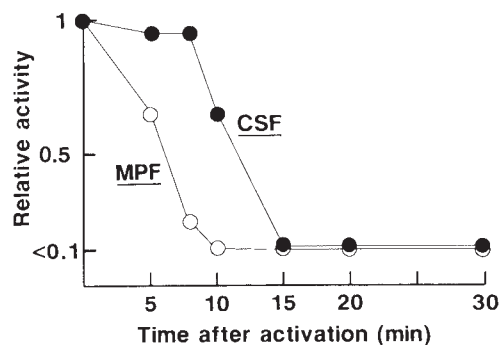


FIG. 1 Degradation kinetics of cyclin B1, B2 and Mos after egg activation. *In vitro* matured eggs prelabelled with ³⁵S-methionine were washed (time 0) and chased either for 30 min without activation (none) or for periods indicated (in min) after activation by pricking (prick). They were then subjected to immunoprecipitation analysis using anti-cyclin B1 antibody (a) or anti-cyclin B2 antibody plus anti-Mos antiserum (b). Identification of cyclins B1, B2 and Mos (indicated by closed arrowheads) was by competition analysis using appropriate antigens (data not shown). The polypeptides indicated by open arrowheads in a are not cyclin B1.

METHODS. Full grown oocytes (stage VI) were obtained and labelled continuously with ³⁵S-methionine (1 mCi ml⁻¹) for 13 h in the presence of progesterone (5 µg ml⁻¹), as described previously¹⁷. The labelled, matured eggs were washed, treated with cycloheximide (300 µg ml⁻¹) for 1 h, activated by pricking with a glass needle and then cultured for periods specified in the presence of cycloheximide. The activated eggs were lysed in radioimmunoprecipitation assay buffer and subjected to immunoprecipitation analysis using either anti-cyclin B1 antibody¹⁵ alone or anti-cyclin B2 antibody¹⁵ plus anti-Mos antiserum, as described previously⁹. The anti-Mos antiserum (polyclonal antibody), was raised against a bacterially expressed Mos protein produced by the vector pET-3a (ref. 23), into which a *NcoI*-*EcoRI* fragment from pSPX-mos (ref. 24) was inserted.

|| To whom correspondence should be addressed.



cyclin B1, disappearing by 15 min after activation (Fig. 1b). This slight but significant difference between the two cyclin species is very similar to that in *Xenopus* embryonic mitosis¹⁶. Figure 1b also shows the degradation kinetics of Mos, which was coanalysed with cyclin B2 (see legend to Fig. 1). In contrast to cyclins, Mos persisted until 15 min after activation, with no appreciable changes in its levels and size. But it underwent a size shift at 20 min and then nearly complete degradation at 30 min after activation. Using ³²P-labelled eggs, we found the size shift to be due to dephosphorylation (data not shown) (Mos is phosphorylated in mature eggs^{9,17}). These results clearly show that the degradation (and even the preceding dephosphorylation) of Mos occurs later than cyclin degradation or MPF inactivation. In both animal and vegetal halves of the activated eggs, we observed a similar difference in the degradation kinetics between Mos and cyclins (data not shown), indicating no regional difference in their degradation kinetics.

These results do not necessarily prove that the inactivation of CSF activity occurs after the inactivation of MPF activity, because the CSF activity (presumably kinase activity^{3,9,18,19}) of Mos protein could be somehow inactivated before its dephosphorylation or degradation. We therefore directly measured both CSF and MPF activities after egg activation. For this, extracts of mature eggs were prepared at appropriate time intervals after activation, and their MPF and CSF activities were assayed by microinjection into immature oocytes and two-cell embryos, respectively^{2,10,20}. MPF activity rapidly decreased after egg activation and reached the basal level at 10 min after activation (Fig. 2). The inactivation kinetics are very similar to those

FIG. 2 Inactivation kinetics of MPF and CSF activities after egg activation. *In vitro* matured eggs were activated by calcium ionophore A23187 and extracts were prepared at indicated times. Activities of MPF and CSF relative to those in nonactivated eggs (time 0) are shown.

METHODS. About 600 eggs matured *in vitro* were activated by treatment with calcium ionophore A23187 (1 µg ml⁻¹). Groups of 80 eggs, each collected at appropriate time intervals after activation, were quickly washed twice with 5 ml chilled extraction buffer (80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl₂ and 1 mM dithiothreitol) and transferred to a 0.5 ml Eppendorf tube. After removing excess buffer, the eggs were crushed by centrifugation (10,000g for 5 min at 2 °C) and clear cytosols (taken in aliquots) were quickly frozen in liquid nitrogen and stored at -80 °C until assay. For MPF activity, 60 nl from each of serially (1–16-fold) diluted cytosol preparations was injected into cycloheximide-treated immature oocytes and the dilution required for 50 % germinal vesicle breakdown in recipient oocytes was determined²⁴. For CSF activity, 30 nl from similarly diluted preparations was injected into two-cell embryos and the dilution required for 50 % 2/4-cell stage arrest was determined²⁰. Both MPF and CSF activities were the highest in the nonactivated egg cytosols and the activities in these extracts were arbitrarily standardized as 1.0.

previously described¹⁰ as well as to the degradation kinetics of cyclins, as shown above. By contrast, CSF activity persisted until 8 min after activation, then decreased to the basal levels at 15 min, showing a good parallel with the dephosphorylation and degradation kinetics of Mos after egg activation (see also Fig. 1b). In three independent experiments, we never observed the precedence of CSF inactivation to MPF inactivation. Thus, these results unequivocally demonstrate that in activated eggs, CSF inactivation does indeed occur later than MPF inactivation. Clearly, this argues strongly that the widely accepted assumption^{2,6,8,10–14}, which postulates the requirement of CSF inactivation for MPF inactivation on meiotic release, is probably wrong. Rather, our results support the novel view that MPF inactivation on meiotic release is independent of CSF inactivation.

Fertilization or egg activation triggers a transient increase in the cytoplasmic free calcium^{4,5} which seems to be required for the inactivation of MPF, because injection of the calcium chelator EGTA into unfertilized *Xenopus* eggs blocks MPF inactivation^{6,21}. Thus, it seems almost certain that the calcium transient on egg activation triggers some other mechanism for MPF inactivation. Indeed, recent studies suggest that inactivation of phosphatase¹⁴ and activation of the ubiquitin pathway²², both of which appear to follow the calcium transient on egg activation, are involved in the inactivation of MPF (Mos or CSF is degraded through the calpain-dependent pathway⁹). We believe that CSF inactivation *per se* serves to allow the fertilized egg to enter successful mitosis because the persistence of CSF (or Mos) after fertilization would cause mitotic arrest of the zygote^{1,2} □

Received 24 April; accepted 10 June 1991.

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ACKNOWLEDGEMENTS. We thank G. F. Vande Woude, Y. Masui and N. Furuno for discussion, K. Okazaki and M. Nishizawa for anti-Mos antiserum, and T. Inoue for assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

Cyclin A and the retinoblastoma gene product complex with a common transcription factor

Lasantha R. Bandara, Jörg P. Adamczewski*,
Tim Hunt* & Nicholas B. La Thangue†

Laboratory of Eukaryotic Molecular Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
* Imperial Cancer Research Fund, Clare Hall Laboratories, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3LD, UK

THE retinoblastoma gene (*Rb*) product is a negative regulator of cellular proliferation¹, an effect that could be mediated in part at the transcriptional level through its ability to complex with the sequence-specific transcription factor DRTF1 (ref. 2). This interaction is modulated by adenovirus E1a, which sequesters the Rb protein³ and several other cellular proteins³, including cyclin A (refs 4, 5), a molecule that undergoes cyclical accumulation and destruction during each cell cycle^{6,7} and which is required for cell cycle progression⁸. Cyclin A, which also complexes with DRTF1, facilitates the efficient assembly of the Rb protein into the complex. This suggests a role for cyclin A in regulating transcription and defines a transcription factor through which molecules that regulate the cell cycle in a negative fashion, such as Rb, and in a positive fashion, such as cyclin A, interact. Mutant loss-of-function *Rb* alleles, which occur in a variety of tumour cells, also fail to complex with E1a and large T antigen^{9,10}. Here we report on a naturally occurring loss-of-function *Rb* allele encoding a protein that fails to complex with DRTF1. This might explain how mutation in the *Rb* gene prevents negative growth control.

DRTF1 is a cellular transcription factor that complexes with the *Rb* gene product in a cell-specific fashion². In JM cells, which are derived from a human T-cell leukaemia, most DRTF1 is complexed to the Rb protein (DRTF1a), whereas in murine F9 embryonal carcinoma (EC) stem cells less Rb is complexed and DRTF1 exists mostly in the dissociated, Rb-free form (DRTF1b). The Rb protein can be sequestered from DRTF1 by adenovirus E1a, an effect that requires E1a conserved regions 1 and 2 (ref. 2), also necessary for the immortalizing activity of E1a (ref. 11). In F9 EC cell extracts DRTF1 DNA-binding activity resolves as three discrete complexes, referred to as a, b and c¹², where DRTF1a migrates more slowly than b and c (from now on collectively referred to as DRTF1b) (Fig. 1, track 2). DRTF1 binds to the same motif as the HeLa cell transcription factor E2F, although the relationship between DRTF1 and E2F is at present unclear.

As DRTF1 complexes with the Rb protein, we tested whether it also complexed with other E1a-associated proteins, several of which have been characterized^{3,13}. One of these is cyclin A (refs 4, 5), a member of the family of molecules first defined in marine invertebrates that undergo cyclic accumulation and destruction during each mitosis of the cell-cycle^{6,7}. Cyclin A has been implicated in the regulation of mitosis from genetic studies⁸ and because of its cyclic accumulation and association with cdc2-related kinases⁵.

To investigate whether cyclin A also complexed with DRTF1, we assessed the effect of an anti-cyclin A polyclonal antibody. Anti-cyclin A antibody, but not the preimmune serum, disrupted DRTF1a, causing it to migrate faster, but had no effect on DRTF1b (Fig. 1, compare tracks 3 and 4; indicated by a'), and neither antibody affected the ATF-site DNA-binding activity

ECRE2 (Fig. 1, compare tracks 7 and 8). The anti-cyclin A antibody caused a faster migrating DRTF1a complex probably by dislodging cyclin A from the transcription factor complex. Two proteins involved in cell cycle control, the Rb protein and cyclin A, are thus present in DRTF1a and both are targeted and sequestered by viral E1a (refs 3-5).

The low level of DRTF1a and high level of DRTF1b in F9 cells could result from limiting amounts of available Rb protein, cyclin A or both. We tested this by expressing and purifying Rb and cyclin A fusion proteins (Fig. 2a), and supplemented F9 cell extracts with each protein before assaying the effect by gel retardation. The Rb fusion protein contained human *Rb* coding sequence from residues 379 and 928, which includes the simian virus 40 large T/E1a binding region^{9,10}, fused to glutathione S-transferase (Fig. 2a); the purification strategy yielded an Rb fusion protein, GT-Rb, free from other contaminating polypeptides (Fig. 2b, track 4).

GT-Rb efficiently complexed with F9 EC DRTF1b because there was an induction of DRTF1a and a concomitant reduction of DRTF1b on addition of GT-Rb to the F9 EC whole cell extract (Fig. 3; compare track 2 with 3, 4 and 5), suggesting that free and appropriately modified Rb protein is limiting and in part responsible for the low level of DRTF1a in F9 EC cells. This also argues that the underphosphorylated form of the Rb protein can assemble into the complex as GT-Rb was purified from prokaryotic cells and should therefore lack the physiologically relevant phosphorylation provided by eukaryotic cells. We

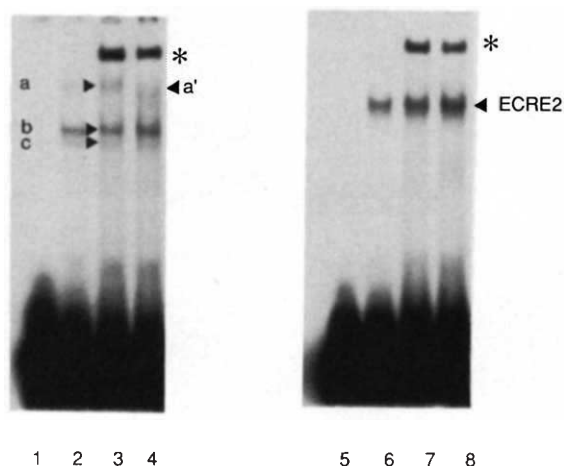


FIG. 1 Cyclin A is complexed to a transcription factor. A rabbit polyclonal antiserum raised against purified bovine cyclin A was added to an F9 EC cell extract and the effect assessed by gel retardation using either a DRTF1 (tracks 1 to 4) or an ATF (tracks 5 to 8) binding site. The F9 EC cell extract alone is shown in tracks 2 and 6, the extract with preimmune rabbit serum in tracks 3 and 7, and with the polyclonal anti-cyclin A in tracks 4 and 8. DRTF1a, b/c and ECRE2 complexes are indicated, a' indicates the faster mobility complex that appears in the anti-cyclin A-treated track (track 4), and asterisks indicate a nonspecific complex caused by the rabbit antisera (tracks 3, 4, 7 and 8). The probes alone are shown in tracks 1 and 5.

METHODS. Gel retardation was performed as described²⁰ in F9 EC whole cell extracts prepared according to standard procedures. The DRTF1 and ATF binding-site probes contained sequences -71 to -50 and -82 to -60, respectively, taken from the adenovirus E2A promoter²⁰. The rabbit polyclonal anti-bovine cyclin A antiserum was prepared by expressing a partial bovine cyclin A complementary DNA clone (J.P.A. and M. Carrington, unpublished results) as a fusion protein in the T7-inducible open reading frame of pET5b. The cyclin A sequence starts at residue 26 (ref. 5) and extends into the 3' nontranslated region. After induction of the fusion protein, inclusion bodies were collected, the fusion protein separated by preparative gel electrophoresis and electroeluted from the gel slice. Rabbits were injected with 4 doses of 200 µg of pure protein at 4-week intervals. The antibody recognizes a single polypeptide with the predicted molecular weight for cyclin A in a wide variety of vertebrate extracts (data not shown).

† To whom correspondence should be addressed.

went on to test whether GT-Rb would associate with affinity-purified DRTF1, which consists predominantly of F9 EC DRTF1b (ref. 12) and contains several polypeptides of relative molecular mass in the range of 50,000 (50K) that bind specifically to the DRTF1 motif, and one of 30K, which binds less efficiently; affinity-purified DRTF1b is a binding site-dependent positively acting transcription factor when its activity is assayed in F9 cell extracts¹². Although GT-Rb efficiently complexed with DRTF1b in F9 EC crude cell extracts, it failed to do so when added to affinity-purified DRTF1b (Fig. 2c, compare tracks 2 and 3 with 6 and 7); but there was a weak interaction between GT-Rb and DRTF1b (Fig. 2c, tracks 6 and 7). This suggested that another molecule present in crude extracts but absent from the affinity-purified fraction was required. An obvious candidate for this was cyclin A because it is present with the Rb protein in DRTF1a (Fig. 1, and ref. 2). To establish whether cyclin A has a role in assembling the Rb protein into DRTF1a, a protein A-cyclin A fusion protein (PA-CA) containing the C-terminal 355 residues (residues 77 to 432) of cyclin A (Fig. 2a) was expressed and purified to homogeneity (Fig. 2b, track 2). PA-CA retains all of the expected mitotic functions of cyclin A so far investigated because it is capable of activating H1 kinase in *Xenopus* egg and HeLa cell extracts (J.P.A. and T.H., manuscript in preparation). Unlike GT-Rb, there was only a marginal effect on DRTF1a when PA-CA was added to crude F9 EC cell extracts (data not shown).

PA-CA did not complex with affinity-purified DRTF1b (Fig. 2c, compare tracks 2 and 3 with 4 and 5), again arguing that another component was required for PA-CA to assemble

efficiently into DRTF1a. But when both GT-Rb and PA-CA were added together, there was a significant enhancement of DRTF1a (Fig. 2c, tracks 8 and 9). This required the activity of both fusion proteins because it did not occur when either of them was denatured (Fig. 2c, tracks 10 to 13), nor was it independent of affinity-purified DRTF1b as the fusion proteins alone had no DNA-binding activity (Fig. 2c, tracks 14 to 15).

Cyclin A activates H1 kinase through its association with cdc2 or cdc2-related kinases⁵. Although it is unlikely that affinity-pure DRTF1b contained such kinases as the purification involved four applications to a binding-site affinity column, we cannot exclude this, so these kinases might have been involved in assembling GT-Rb and PA-CA into DRTF1a. This possibility was, however, extremely unlikely because GT-Rb, PA-CA and DRTF1b assembled in the absence of ATP and, moreover, the assembly was not affected by including a monoclonal antibody against the conserved PSTAIRE motif in cdc2 and related kinases (data not shown). Cyclin A therefore facilitates the assembly of the Rb protein into DRTF1a, a role which is independent of cdc2 kinase. We believe that cyclin A facilitates the assembly of the Rb protein, rather than the converse, because there was weak binding between GT-Rb and DRTF1b but not between PA-CA and DRTF1b (Fig. 2, compare tracks 4 and 5 with 6 and 7).

The *Rb* gene is frequently mutated in a variety of tumour cells to produce loss-of-function alleles, either through point mutation or deletion^{14,15}. These mutants simultaneously lose the ability to complex with E1a and SV40 large T antigen^{9,10}. It has therefore been proposed that mutant Rb proteins can no longer

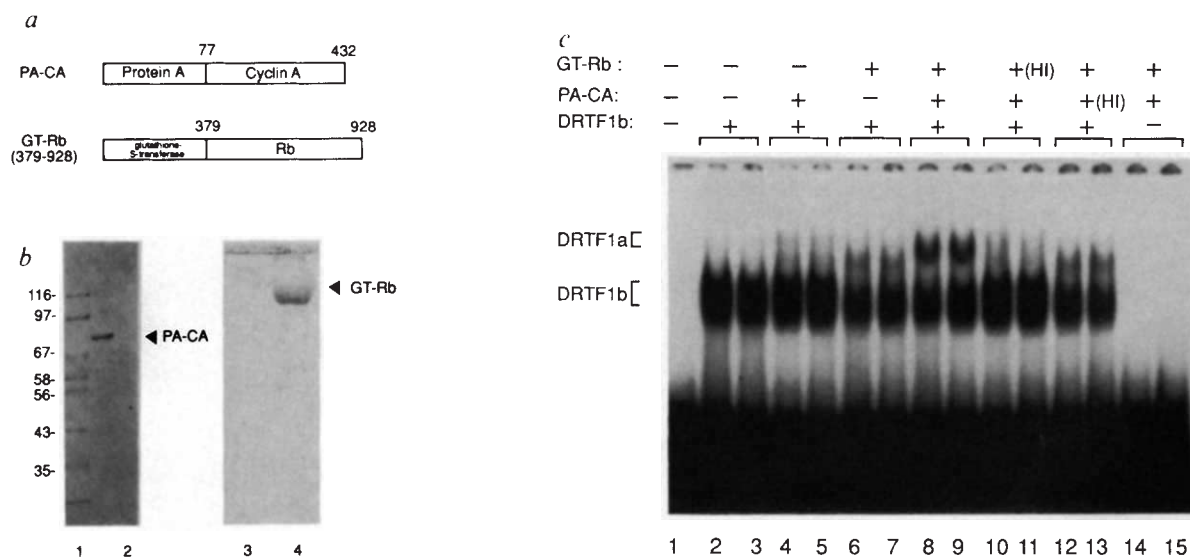


FIG. 2 Cyclin A is necessary for the Rb protein to complex efficiently with DRTF1b. **a**, Diagram of glutathione S-transferase-Rb (GT-Rb) and protein A-cyclin A (PA-CA) fusion proteins. pGT-Rb contains human Rb sequence from residues 379 to 938, and pPA-CA, the C-terminal 355 residues of bovine cyclin A from residues 77 to 432. **b**, Affinity-purified PA-CA (track 2) and GT-Rb (track 4) fusion proteins. Track 1 shows molecular mass markers (K) for track 2, and track 3 shows a control eluate from uninduced pGT-Rb-containing bacteria. The relative molecular mass of GT-Rb is ~80K. **c**, Either affinity-purified PA-CA (~1 µg) or GT-Rb (~150 ng) was added alone (tracks 4 and 5, and 6 and 7, respectively) or together (tracks 8 and 9) to affinity-purified F9 EC DRTF1b (~5 ng; shown alone in tracks 2 and 3). Either GT-Rb or PA-CA was denatured by heat-inactivation (HI) and added to native PA-CA (tracks 10 and 11) or GT-Rb (tracks 12 and 13), together with affinity-purified DRTF1b; there was no DNA-binding activity of the fusion proteins alone (tracks 14 and 15). Note that there was very weak binding between GT-Rb and DRTF1b, and that this was enhanced when both fusion proteins were added together (indicated by 1a in tracks 8 and 9). **METHODS.** The protein A-cyclin A fusion protein vector (pPA-CA) was constructed by isolating the 1.6-kilobase fragment encoding the C-terminal

355 residues of bovine cyclin A and ligating this in-frame with the protein A gene from pRIT2T (Pharmacia), cloned into pET3a, enabling expression to be regulated from the T7 promoter. This fusion protein comprises the N-terminal 265 residues of protein A followed by the C-terminal 355 residues of cyclin A. After induction of the fusion protein the extract was centrifuged at 60,000 r.p.m. for 15 min, precipitated with ammonium sulphate, dissolved in 20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM DTT and fractionated over an ACA-34 gel filtration column. Fractions were assayed by SDS-PAGE, the peak fractions pooled and loaded onto a Mono-Q column; protein was eluted with a linear gradient to 1 M NaCl. The pooled peak fractions were dialysed extensively against 10 mM Tris-HCl, pH 8.0, 1 mM DTT. pGT-Rb contains human Rb coding sequence (residue 379 to 928) cloned into the glutathione S-transferase fusion protein vector pGEX-2T as described²¹. After induction, GT-Rb was purified by glutathione S-transferase affinity chromatography and eluted with reduced glutathione²². DRTF1b was purified from F9 EC whole cell extracts as before by applying four times to the binding-site matrix¹². Gel retardation and binding-site probes as in Fig. 1. Fusion proteins were added to affinity-pure DRTF1b and preincubated for 10 min at 30 °C before addition of the binding site.

complex with a cellular protein, which is predicted to have some degree of homology with the Rb-binding domains in E1a and large T antigen and is necessary for the wild-type Rb protein to mediate its growth-suppressing effects. Because DRTF1 is complexed with the wild-type Rb protein *in vivo*² and as this complex is destroyed by E1a (ref. 2) and SV40 large T antigen (data not shown), we reasoned that mutation in the *Rb* gene may also compromise the DRTF1-Rb interaction. We tested this using the *in vitro* Rb assembly assay in which GT-Rb, containing wild-type sequence, efficiently complexed with DRTF1b (Fig. 3). The assembly of GT-Rb was compared with GT-Rb⁷⁰⁶, which contains the same sequence as GT-Rb apart from a single cysteine → phenylalanine substitution at residue 706 (ref. 14). This natural mutation occurred in a small cell lung carcinoma and no longer binds E1a or large T antigen¹⁴. GT-Rb induced DRTF1a efficiently (Fig. 3, compare track 2 with 3, 4 and 5), whereas there was no effect on addition of GT-Rb⁷⁰⁶ (Fig. 3, compare track 2 with 6, 7 and 8). The single amino-acid change in GT-Rb⁷⁰⁶ therefore produces an Rb protein incapable of complexing with DRTF1.

The wild-type *Rb* gene product is a negative regulator of cellular proliferation, an effect that might be mediated at the transcriptional level through its ability to regulate the activity of transcription factors such as DRTF1 (ref. 2). As the underphosphorylated form of the Rb protein is in DRTF1a, and given that the transcriptional activity of Rb-complexed DRTF1 may be compromised², an important mechanism for Rb-mediated growth control might involve the conversion of active transcription factors to their inactive counterparts. A naturally occurring mutation in the *Rb* gene, which changes a single amino acid in the wild-type sequence¹⁴, encodes an Rb protein that cannot interact with DRTF1, arguing that DRTF1 is important for wild-type Rb to mediate its normal cellular function of negative growth control. Loss-of-function *Rb* alleles also fail to bind to E1a or large T (refs 9, 10, 14). It is likely, therefore, that the normal function of Rb is mediated through a protein that has a region with structural similarity to the Rb-binding domain in E1a and large T. We propose DRTF1 as a candidate for such a molecule.

Both cyclin A and the Rb protein locate to DNA by virtue of an ability to complex with DRTF1, suggesting that cyclin A might have a role in regulating transcription. Cyclin A also

functions to assemble the Rb protein efficiently into the transcription factor complex, an effect independent of cdc2 and cdc2-like kinases. It is still possible that dissociation of the complex is dependent on these kinases because the activity of cdc2 is activated when it complexes with cyclin A⁵. In this respect, DRTF1-associated cyclin A may serve two separate functions: first, to guide cdc2 to key substrates, such as the Rb protein in DRTF1, and second, to activate cdc2 kinase once it has located its substrate. It is consistent with such a model that the Rb protein is efficiently phosphorylated by human cdc2 kinase¹⁶. Given that the underphosphorylated form of the Rb protein complexes with DRTF1, the form thought to mediate its growth regulating properties¹⁷⁻¹⁹, phosphorylation may release the Rb protein from DRTF1a and so enable DRTF1b to activate transcription of target genes. □

Received 5 June; accepted 27 June 1991.

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ACKNOWLEDGEMENTS. We thank W. Kaelin and D. Livingston for pGT-Rb³⁷⁹⁻⁹²⁸ and pGT-Rb⁷⁰⁶, M. Carrington for help with the production of the anti-cyclin A antibody, J. Minshull for construction of the T7 protein A expression vector, P. Rigby for critically reading the manuscript, and M. K. H. Kumar for discussion. J.P.A. acknowledges receipt of a graduate scholarship from the Stiftung Stipendienfonds des V.C.I. e.V., and L.R.B. is funded by an MRC Collaborative Studentship with Roche Products. N.B.L.T. is a Jenner Fellow of the Lister Institute of Preventive Medicine.

Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product

Deborah Defeo-Jones, Pearl S. Huang,
Raymond E. Jones, Kathleen M. Haskell,
Gerald A. Vuocolo, Michelle G. Hanobik,
Hans E. Huber & Allen Oliff*

Department of Cancer Research, Merck Sharp and
Dohme Research Laboratories, West Point, Pennsylvania 19486, USA

THE E7 transforming protein of human papilloma virus-16 binds to the retinoblastoma gene product (pRb)^{1,2} through a nine-amino-acid segment of E7 (21-29)³⁻⁵. This segment of E7 is homologous to the pRb-binding domains of the simian virus 40 large T and adenovirus E1A transforming proteins⁶⁻⁹. Each of these viral transforming proteins bind to the same region of pRb^{10,11}. To isolate cellular proteins that interact with this viral protein-binding domain on pRb^{12,13}, we used recombinant pRb to screen a human complementary DNA expression library. Two cDNAs were isolated that encode retinoblastoma binding proteins (RBP-1 and RBP-2). We report here that these RBP genes exist in separate loci and

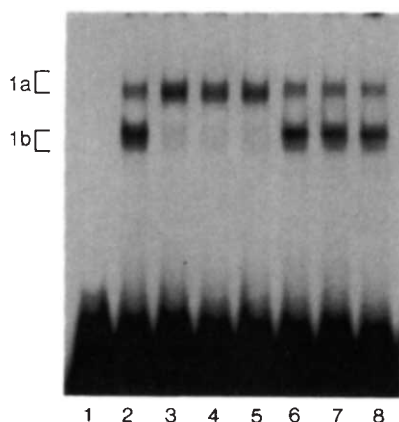


FIG. 3 A naturally occurring mutant Rb protein, Rb⁷⁰⁶, does not complex with DRTF1. Either affinity-purified GT-Rb (tracks 3, 4 and 5) or GT-Rb⁷⁰⁶ (tracks 6, 7 and 8) fusion proteins were added in increasing amounts (~45 ng, 75 ng and 105 ng, respectively) to an F9 EC cell extract (shown alone in track 2) and the effect assessed using a DRTF1 binding site probe; track 1 shows the probe alone.

METHODS. GT-Rb and GT-Rb⁷⁰⁶ were expressed and purified as described in the legend to Fig. 2. Fusion proteins were preincubated with the extract for 10 min before addition of the binding site probe. Binding site probes are described in Fig. 2 legend.

* To whom correspondence should be addressed.

produce discrete messenger RNAs. The predicted amino-acid sequence of these genes showed no homology to known proteins, but both RBPs contain the pRb binding motif conserved between E7, large T and E1A¹⁴. *In vitro* expression of the RBP cDNAs yielded proteins that specifically bound to pRb. Recombinant E7 protein, the E7 21–29 peptide and the homologous RBP-1 peptide inhibited RBP–pRb binding. Mutations introduced into the putative pRb-binding segment in RBP-1 impaired its binding activity. These studies indicate that the cellular RBP-1, RBP-2 and viral E7 proteins interact with pRb through similar domains.

A version of recombinant pRb (pRb60) of relative molecular mass 60,000 (*M*_r 60K) containing the viral transforming protein-binding domain^{10,11} was produced in bacteria and isolated using affinity chromatography (manuscript in preparation). To ensure that the pRb obtained from this isolation scheme was able specifically to bind to HPV-16 E7, an E7 21–29 peptide capture column was used as the final purification step. Recombinant pRb protein that bound to this column was eluted in a low-salt buffer at high pH. The pRb produced in this manner saturated the binding sites for recombinant human papilloma virus (HPV-16) E7 protein in microtitre plate binding assays⁵ and exhibited only minimal nonspecific binding. A λ gt11 human lung fibroblast cDNA expression library (Clontech, Palo Alto, California) was probed using the peptide column-purified pRb. Unbound pRb was eliminated by extensive washing with binding buffer^{15,16}. Bacteriophage plaques that bound pRb were identified using a cocktail of anti-pRb murine monoclonal antibodies (E. Harlow, Boston, Massachusetts). Alkaline phosphatase-conjugated goat anti-murine IgG antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania) provided a chromogenic identification of the monoclonal antibody-positive plaques. After several rounds of plaque purification, recloning and repeat verification of pRb binding, two independent clonal isolates were obtained and designated retinoblastoma binding proteins (RBP) RBP-1 and RBP-2.

The cDNA inserts from each phage isolate were excised and transferred into a modified transcription plasmid vector pGEM

3Z for further analysis. This modified vector inserted an ATG start codon at the 5' end of the cDNA coding sequences. Each cDNA insert was radiolabelled and used in turn to probe unlabelled insert DNA from the other clone. No evidence of cross-hybridization was found between the RBP-1 and RBP-2 cDNAs. The radiolabelled cDNAs from RBP-1 and RBP-2 were next used to probe human cellular DNA and mRNA in Southern and northern blot transfer experiments. Figure 1 shows the restriction enzyme fragments identified by the radiolabelled RBP probes. The limited number of hybridization-positive cellular DNA bands evident even after prolonged exposures suggest that RBP-1 and RBP-2 are encoded by single copy genes. Additional examinations of cellular DNA blots from other species (mouse, cow, monkey, chicken and rabbit), examined under conditions of reduced-stringency hybridization, indicated that these genes are conserved among all species tested (data not shown). RNA analyses of RBP-1 and RBP-2 used mRNAs isolated from normal human lung tissue. The RBP-1 probe identifies two messages of about 5.2 and 4.3 kilobases (kb) (Fig. 2). The RBP-2 probe identifies a single message of about 6.0 kb. These studies indicate that RBP-1 and RBP-2 are actively transcribed in normal human cells.

The RBP-1 and RBP-2 cDNA clones were sequenced using the dideoxy nucleic-acid sequencing method¹⁷. The complete sequence of each clone is shown in Fig. 3*a, b*. The RBP-1 clone contains an open reading frame of 348 amino acids. RBP-2 contains an open reading frame of 464 amino acids. These amino-acid sequences represent the open reading frames from each clone that were consistent with the LacZ fusion proteins encoded by the λ gt11 RBP-1 and RBP-2 cDNAs¹⁸. Both amino-acid sequences were distinct from one another. No homology to known proteins was found in either RBP-1 or RBP-2 when their sequences were compared with the GENEMBL DNA sequence data base using the TFASTA algorithm¹⁹. Most notably, both RBP clones contain a stretch of 10 amino acids that mimics the pRb-binding domain of HPV-16 E7 (Fig. 3*c*). The pRb-binding domains of E1A, SV40 large T, HPV-16, HPV-18 and cottontail rabbit papilloma virus (CRPV) are shown for

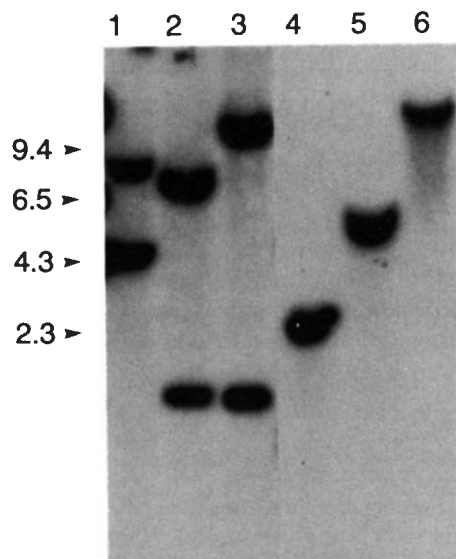


FIG. 1 Southern blot transfer analysis of RBP-1 and RBP-2 sequences in human placental DNA. Agarose gel wafers containing restricted human placenta DNA (Clontech) were hybridized with full-length RBP-1 or RBP-2 cDNAs. DNA probes were labelled with ³²P using a Hexamer-prime Kit (Boehringer Mannheim, Indianapolis, Indiana) according to the manufacturer's instructions. After washing with 0.1×SSC buffer at 65 °C, filters were autoradiographed for 15 h. Band sizes were estimated using *Hind*III cut λ DNA markers as indicated at the left margin. DNA was restricted with *Eco*RI (lanes 1 and 4), *Hind*III (lanes 2 and 5), *Bam*HI (lanes 3 and 6). Lanes 1–3 were probed with RBP-2 cDNA, lanes 4–6 were probed with RBP-1 cDNA.

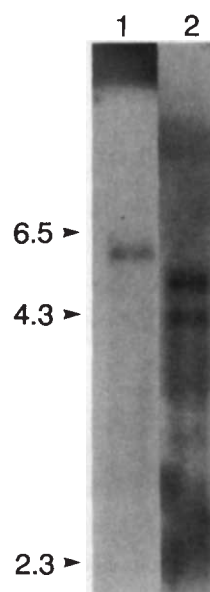


FIG. 2 Northern blot transfer analysis of RBP-1 and RBP-2 sequences in human lung tissue mRNA. Poly(A)-selected RNA (5 μ g) from normal human lung tissue (Clontech) was electrophoresed in each lane of a 0.8% agarose-formaldehyde gel, transferred to nitrocellulose filter paper and probed with RBP-1 or RBP-2 ³²P-labelled cDNAs made using a Hexamer-prime Kit (Boehringer Mannheim). Hybridization and wash conditions were as previously described²⁵. RBP-1 probe (lane 2) and RBP-2 probe (lane 1).

a

RBP-1

GGC CGC ATG AGC CCG TCA GTA TCG GCG GAA TTC CGG TTG AAA CGG AAA ATA CTA GGA CAA
 - - - - - Leu Lys Arg Lys Ile Leu Gly Gln

TCA TCG CCA GAG AAA AAA ATA AGA ATT GAG AAT GGA ATG GAA ATG ACA AAT ACT GTA TCT
 Ser Ser Pro Glu Lys Lys Ile Arg Ile Glu Asn Gly Met Glu Met Thr Asn Thr Val Ser

CAA GAA AGG ACC AGT GAT TGT ATT GGA TCT GAG GGA ATG AAA AAC TTA AAT TTT GAA CAG
 Gln Glu Arg Thr Ser Asp Cys Ile Gly Ser Glu Gly Met Lys Asn Leu Asn Phe Glu Gln

CAC TTT GAA AGA GAA AAT GAA GGA ATG CCA TCA TTG ATA GCA GAG TCA AAC CAA TGC ATC
 His Phe Glu Arg Glu Asn Glu Gly Met Pro Ser Leu Ile Ala Glu Ser Asn Gln Cys Ile

CAA CAA CTG ACT AGT GAA AGA TTT GAT AGT CCA GCT GAA GAA ACT GTA AAT ATT CCA CTA
 Gln Gln Leu Thr Ser Glu Arg Phe Asp Ser Pro Ala Glu Glu Thr Val Asn Ile Pro Leu

AAA GAA GAT GAG GAT GCA ATG CCT CTG ATC GGG CCT GAA ACC TTG GTT TGC CAT GAA GTA
 Lys Glu Asp Glu Asp Ala Met Pro Leu Ile Gly Pro Glu Thr Leu Val Cys His Glu Val

GAT TTG GAT GAT TTG GAT GAA AAG GAT AAG ACC AGC ATT GAG GAT GTA GCA GTT GAA AGC
Asp Leu Asp Asp Leu Asp Glu Lys Asp Lys Thr Ser Ile Glu Asp Val Ala Val Glu Ser

TCT GAG TCT AAC TCT CTT GTT TCT ATT CCA CCT GCC CTA CCT CCT GTA GTC CAA CAT AAC
 Ser Glu Ser Asn Ser Leu Val Ser Ile Pro Pro Ala Leu Pro Pro Val Val Gln His Asn

TTT TCA GTA GCT TCA CCA CTT ACT CTT AGT CAA GAT GAG TCT CGA AGC GTA AAA AGT GAG
 Phe Ser Val Ala Ser Pro Leu Thr Leu Ser Gln Asp Glu Ser Arg Ser Arg Ser Ile Ile Val

AGT GAT ATA ACG ATT GAA GTT GAT AGT ATT GCT GAA GAA TCT CAA GAA GGT CTC TGT GAG
 Ser Asp Ile Thr Ile Glu Val Asp Ser Ile Ala Glu Glu Ser Gln Glu Gly Leu Cys Glu

AGG GAA TCG GCA AAT GGA TTT GAA ACT AAT GTT GCC TCT GGT ACC TGT AGT ATA ATT GTA
 Arg Glu Ser Ala Asn Gly Phe Glu Thr Asn Val Ala Ser Gly Thr Cys Ser Ile Ile Val

CAA GAG AGA GAG AGC AGA GAG AAG GGT CAG AAG AGG CCA AGT GAT GGA AAT AGT GGA TTA
 Gln Glu Arg Glu Ser Arg Glu Pro Ser Asp Gly Asn Ser Gly Leu

ATG GCA AAA AAG CAA AAG CGT ACC CCA AAG CGA ACA AGT GCT GCA GCC AAA AAT GAA AAG
 Met Ala Lys Lys Gln Lys Arg Thr Pro Lys Arg Thr Ser Ala Ala Ala Lys Asn Glu Lys

AAT GCA ACA GGA ACA AGC AGT GAT AGT GAA GAT CTC CTT GTC CTA GAC AAT TCA AGT AAA
 Asn Gly Thr Gly Gln Ser Ser Asp Ser Glu Asp Leu Pro Val Leu Asp Asn Ser Ser Lys

TGT ACC CCA GTA AAG CAT CTT AAT GTA TCT AAG CCA CAG AAA CTT GCA CGA TCT CTT GCA
 Cys Thr Pro Val Lys His Leu Asn Val Ser Pro Gln Lys Leu Ala Arg Ser Ile Ile Pro Ala

AGA ATA TCC CCG CAC ATC AAA GAT GGA GAG AAA GAT AAA CAC AGA GAA AAA CAT CCG AAT
 Arg Ile Ser Pro His Ile Lys Asp Gly Glu Lys Asp Lys His Arg Glu Lys His Pro Asn

TCA TCC CTT AGG ACA TAT AAA TGG AGC TTT CAG CTC AAT GAA TTG AGT AAT ATG AAC AGT
 Ser Ser Pro Arg Thr Tyr Lys Trp Ser Phe Gln Leu Asn Glu Leu Ser Asn Met Asn Ser

ACA GAG AGA ATC TCA TTT CTC CAA GAA AAA CTA CAG GAA TCA GAA AAT ATT ATA TGT CTT
 Thr Glu Arg Ile Ser Phe Leu Gln Glu Lys Leu Gln Glu Ser Glu Asn Ile Ile Cys Leu

TGA AGT CTA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA
 * Ser Leu

b

RBP-2

GAA AAG GAT CTG GAC CTG GAG CCT CTG AGT GAT CTG GAG GAA GGA TTG GAG GAA ACC AGA
 Glu Lys Asp Leu Asp Lys Glu Pro Lys Ser Asp Leu Glu Glu Gly Leu Glu Glu Thr Arg

GAT ACA GCC ATG GTG GTG GCA GTT TTT AAA GAA CGG GAG CAA AAA GAG ATT GAA GCC ATG
 Asp Thr Ala Met Val Val Ala Val Phe Lys Glu Arg Glu Gln Lys Glu Ile Glu Ala Met

CAT TCT CTC AGA GCA GCC AAC CTA GCC AAG ATG ACA ATG GTG GAC CGC ATA GAA GAA GTA
 His Ser Thr Pro Val Lys Ala Ala Asn Leu Ala Lys Met Thr Met Val Asp Arg Glu Arg Leu

AAA TTT TGC ATT TGC CCG AAG ACA GCC AGT GGG TTT ATG CTA CAG TGT GAG CTC TGC AAA
 Lys Phe Cys Ile Cys Arg Lys Thr Ala Ser Gly Phe Met Leu Gln Cys Glu Leu Cys Lys

GAC TGG TTC CAT AAC AGC TGT GTT CCT CTT CCA TCA AGT TCC CAA AAA AAA GGA TCC
 Asp Trp Phe His Asn Ser Cys Val Pro Leu Pro Lys Ser Ser Ser Gln Lys Lys Gly Ser

AGC TGG CAA GCT AAA GAA GTA AAA TTC CTT TGC CCT CTT TGT ATG CGG TCT CGA AGG CCC
 Ser Trp Gln Ala Lys Glu Val Lys Phe Leu Cys Pro Leu Lys Met Arg Ser Arg Arg Leu

AGG CTA GAG ACT ATT CTG TCA CTC CTG GTA TCC CTT CAG AAG TTG CCC GTA CGG TTG CCT
 Arg Leu Glu Thr Ile Leu Ser Leu Lys Leu Val Ser Leu Gln Lys Leu Pro

GAA GGA GAG GCC CTG CAG TGT TTG ACA GAA CGT GCT ATG AGT TGG CAA GAT AGA GCG CGG
 Glu Gly Glu Ala Leu Gln Cys Leu Thr Glu Arg Ala Met Ser Trp Gln Arg Arg Ala Arg

CAG GCT CTA GCC ACA GAT GAA CTA TCC TCT GCC CTC GCC AAA CTA TCT GTG TTG AGC CAG
 Gln Ala Leu Ala Thr Asp Glu Leu Ser Ser Ala Leu Ala Lys Leu Ser Val Leu Ser Gln

CGT ATG GTG GAA CAG GCG GCT CGA GAA AAA ACT GAA AAG ATC ATC AGT GCA GAA CTC CAA
 Arg Met Val Glu Glu Ala Ala Arg Glu Lys Thr Glu Lys Ile Leu Ser Ala Glu Glu Lys

AAA GCA GCT GCC AAT CCA GAC TTA CAG GGA CAC TTA CCT AGT TTC CAG CAG TCT GCT TTT
 Lys Ala Ala Ala Asn Pro Asp Leu Gln Gly His Leu Pro Ser Phe Gln Gln Ser Ala Phe

AAC CGG GTG GTG AGC AGT GTG TCA TCT CTT CCG CAA ACA ATG GAC TAT GAT GAT GAA
 Asn Arg Val Val Ser Ser Val Ser Ser Ser Pro Arg Gln Thr Met Asp Tyr Asp Asp Glu

GAA ACA GAC TCT GAT GAA GAC ATT CGA GAG ACA TAT GGC TAC GAC ATG AAG GAC ACA GCC
 Glu Thr Asp Ser Asp Glu Asp Ile Arg Glu Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

AGT GTG AAG TCC TCT AGT AGT CTT GAA CCC AAT CTT TTT TGT GAT GAA GAT ATT CCC ATC
 Ser Val Lys Ser Ser Ser Ser Ser Leu Glu Pro Asn Leu Phe Cys Asp Glu Glu Ile Pro Ile

AAA TCC GAG GAG GTG GTG ACC CAC ATG TGG ACA GCA CCT TCA TTT TGT GCA GAG CAT GCT
 Lys Ser Glu Glu Val Val Thr His Met Trp Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr

TAT TCT TCT GCT TCT TCT AAG AGT TGT TCT CAA GTA TTT TTT GGG AAA GGT TCT AGC ACC CCA
 Tyr Ser Ser Ala Ser Lys Ser Cys Ser Gln Val Phe Phe Gly Lys Gly Ser Ser Thr Pro

AGG AAA CAA CCT CGG AAG AGC CCT TTG GTG CCC GAA AGT TTG GAA CCT CCA GTG CTG GAG
 Arg Lys Gln Pro Arg Lys Ser Pro Leu Val Pro Arg Ser Leu Glu Pro Pro Val Leu Glu

TTG TCA CCT GGA GCT AAG GCA CAA CTG GAA GAA CTT ATG ATG GTT GGA GAT CTC CTG GAA
 Leu Ser Pro Gly Ala Lys Ala Gln Leu Glu Glu Met Met Val Gly Asp Leu Leu Glu

GTA TCT CTG GAC GAG ACT CAA CAC ATA TGG CGG ATT TTG CAG GCC ACA CCA CCC TCT
 Val Ser Leu Asp Glu Thr Gln His Ile Trp Arg Ile Leu Gln Ala Thr His Pro Pro Pro

GAA GAG AGA TTT TTG CAT ATC ATG GAG GAT GAC AGC ATG GAA GAG AAA CCA CTA AAA GTG
 Glu Asp Arg Phe Leu His Ile Met Glu Gln Lys Ser Met Glu Glu Lys Pro Leu Lys Val

AAA GGA AAG GAC TCT TCA GAG AAG AAA CGG AAA CGG AAG CTA GAA AAG GTA GAG CAA CTT
 Lys Glu Lys Asp Ser Ser Ser Ser Lys Lys Arg Lys Arg Lys Glu Lys Val Glu Gln Leu

TTT GGA GAA GGA AAA CAG AAG TCC AAG GAG TTA AAG AAA ATG GAC AAA CCT AGA AAG AAG
 Phe Gly Glu Gly Lys Gln Lys Ser Lys Glu Lys Lys Lys Met Asp Lys Pro Arg Lys Lys

AAA TTA AAA TTA GGT GCA GAC AAA TCA AAG AAG CTG AAT AAA CTG GCC AAG AAA CTA GCA
 Lys Leu Lys Leu Gly Ala Asp Lys Ser Lys Lys Leu Asn Lys Leu Ala Lys Lys Leu Ala

AAA AAA AAA AAA
 Lys Lys Lys Lys

comparison. Additionally, RBP-1 and RBP-2 contain casein kinase II consensus sequences close to their pRb-binding domains. The casein kinase II consensus sequence is also a highly conserved genetic element present in the transforming proteins of adenovirus, SV40, HPV and CRPV.

To prove that the RBP cDNAs encoded pRb-binding proteins, each cDNA was subcloned into a modified pGEM vector and used to prepare *in vitro* transcribed mRNA followed by *in vitro* translation of each RBP protein using rabbit reticulocyte lysates. The RBP proteins produced in this system were radiolabelled by incorporation of ^{35}S -labelled methionine. The pRB60 protein was produced in bacteria as a fusion protein with glutathione S-transferase (GST) using the pGEX expression plasmid²⁰ as described by Livingston *et al.*¹² The GST-pRB60 fusion protein was noncovalently bound to glutathione coupled to sepharose beads. The GST-pRB60-bound sepharose beads were then used to test for specific binding to the radiolabelled RBP proteins. As seen in Figure 4a, HPV-16 E7 protein, RBP-1 protein and RBP-2 protein all bound to GST-pRB60 (lane 0) but were partially inhibited from binding by either full-length E7 protein (lane 1) or the E7 21-29 peptide (lane 2). A scrambled E7 peptide consisting of the same residues present in E7 21-29 but out of sequence did not affect RBP binding even when used at 10 times the concentration of E7 21-29 that inhibited RBP binding to GST-pRB60 (lane 3). The homologous peptide from RBP-1 consisting of amino-acid residues ETLVCHEVDL (Fig. 3c, single-letter amino-acid code) also inhibited binding to GST-pRB60, albeit to a lesser degree than the E7 21-29 peptide (lane 4). Two mutant forms of RBP-1 were created and tested for GST-pRB60 binding. RBP-1* is a deletion mutant missing 75 amino-acid residues, including the entire putative viral pRb-binding domain shown in Fig. 3c. Although the RBP-1* construct produced similar quantities of radiolabelled protein as RBP-1 following *in vitro* translation (data not shown), virtually none of this protein bound to GST-pRB60. RBP-1** is a point mutant containing a single amino-acid change of cysteine to alanine in the putative pRb-binding domain. The homologous change of cysteine to alanine or glycine at position 24 of HPV-16 E7 has been shown to reduce pRb binding and impair transformation

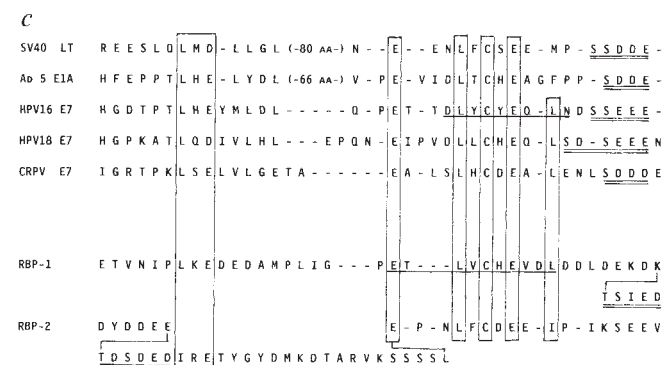


FIG. 3 Nucleotide and deduced amino-acid sequences of RBP-1 and RBP-2 cDNAs. **a**, RBP-1 sequence. The first 12 codons were contributed by Agt11 sequences. The first RBP-1 codon is TTG encoding leucine. The underlined sequence encodes the region of RBP-1 homologous with the E7 21-29 peptide. **b**, RBP-2 sequence. The first RBP-2 codon is GAA encoding glutamic acid. The underlined sequence encodes the region of RBP-2 homologous with the E7 21-29 peptide. **c**, Amino-acid sequence comparison of RBP-1, RBP-2 and the pRb-binding domains of SV40 large T, adenovirus 5 E1A, HPV-16 E7, HPV-18 E7 and CRPV E7 proteins. Boxed residues indicate regions of strong homology. The boxed residues on the left include part of the domain involved in E1A binding to p300 (ref. 26). Boxed residues on the right include conserved residues needed for pRb binding. Singly underlined residues represent sequences of synthetic peptides using in competition experiments (Fig. 4). Doubly underlined residues represent casein kinase II consensus sequences.

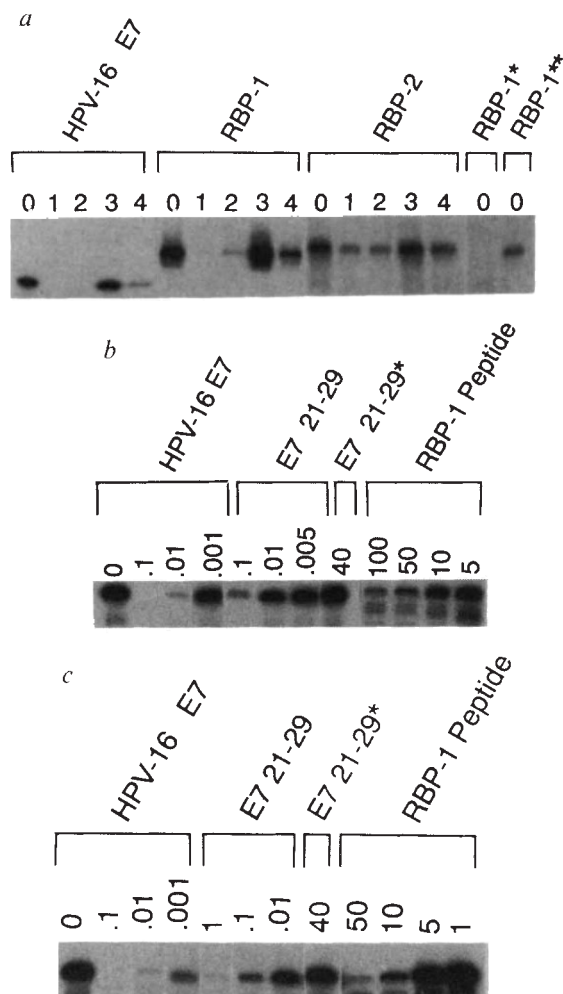


FIG. 4 RBP-specific binding to pRb. *a*, 35 S-labelled methionine HPV-16 E7, RBP-1, RBP-2, RBP-1* or RBP-1** proteins were incubated with GST-pRB60 protein that was noncovalently coupled to glutathione-Sepharose 4B (Pharmacia, Sweden). The incubation was done in the presence or absence of competitor proteins or peptides: lane 0, no competitor; 1, 1 μ M HPV-16 E7 protein; 2, 5 μ M E7 21–29 peptide; 3, 50 μ M scrambled E7 21–29* peptide; 4, 50 μ M of RBP-1 peptide. A faint band with increased electrophoretic mobility consistent with the decreased size of RBP-1* protein is evident after prolonged exposure of the autoradiograph. GST-fusion proteins were coupled to sepharose beads as described¹². The pGEM constructs of pRB60, HPV-16 E7, RBP-1, RBP-2, RBP-1* and RBP-1**, were used to generate *in vitro* synthesized mRNAs. *In vitro* transcription and translations in a rabbit reticulocyte translation system in the presence of 35 S-labelled methionine, $>1,000$ Ci mmol⁻¹ (Amersham), were performed according to the manufacturers instructions (Promega, Madison, Wisconsin). The binding reactions were done in 50 mM HEPES, pH 7.0, 250 mM NaCl and 0.1% Nonidet P-40. An equal number of TCA-precipitable counts of each radiolabelled *in vitro* translation product were added to each reaction. The translation products of each reaction exhibited one predominate band containing $\geq 90\%$ of the incorporated label. Peptides were prepared as described⁶, and were acetylated at the N terminus and amidated at the C terminus. *b*, 35 S-labelled methionine pRB60 was incubated with a GST-RBP-1 fusion protein that was noncovalently coupled to glutathione-Sepharose 4B. The incubation was done in the presence of HPV-16 E7 protein, E7 21–29 peptide, E7 21–29* scrambled peptide or the RBP-1 peptide at the concentrations indicated. The concentration of each competitor is given in μ mol l⁻¹. *c*, 35 S-labelled methionine pRB60 was incubated with a GST-RBP-2 fusion protein that was noncovalently coupled to glutathione-Sepharose 4B. The incubation was performed in the presence or absence of the same competitors described above. Roughly equivalent amounts of GST-pRB60, GST-RBP-1 and GST-RBP-2 proteins were used in each reaction by normalizing the amount of beads added to each reaction after determining the quantity of fusion protein associated with a fixed quantity of Sepharose beads.

activity^{21–24}. RBP-1** exhibited reduced binding to GST-pRB60 relative to RBP-1.

To establish further the ability of RBP-1 and RBP-2 to compete with HPV-16 E7 for binding to pRB60, GST-RBP-1 and GST-RBP-2 fusion proteins were constructed and bound to glutathione-Sepharose beads. *In vitro* translated and radiolabelled pRB60 was bound to GST-RBP-1 (Fig. 4b) or GST-RBP-2 (Fig. 4c) in the presence of varying concentrations of recombinant HPV-16 E7 protein, E7 21–29 peptide, scrambled E7 21–29* peptide, or the RBP-1 peptide (ETLVCHEVDL). In each case increasing concentrations of E7 protein, E7 21–29 peptide, and RBP-1 peptide progressively diminished pRB60 binding to GST-RBP-1 and GST-RBP-2, whereas the scrambled E7 21–29* peptide has no apparent effect on binding. Interestingly, E7 protein was more potent than the E7 21–29 peptide which in turn was more potent than the RBP-1 peptide at inhibiting

binding of either RBP-1 or RBP-2 to pRB60. This high affinity of E7 binding to pRb may have evolved to enable E7 to dominate the normal interactions of cellular proteins with pRb.

The RBPs identified here seem to be human cellular homologues of the DNA tumour virus-transforming proteins E7, E1A and large T. Our studies suggest that the RBPs bind in the same 'pocket' domain of pRb as HPV-16 E7 and the E7 21–29 peptide. The finding of an E7-like pRb-binding domain in both RBPs and the critical nature of this domain for pRb binding suggests that the RBPs use the same binding motif as E7, E1A and large T to interact with pRb. The biochemical similarity between the RBPs and HPV-16 E7 in pRb binding suggests that these proteins may have similar functional properties in mammalian cells. Studies are in progress to determine whether either of the RBPs can modulate cell growth properties *in vitro*. □

Received 28 May; accepted 25 June 1991.

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ACKNOWLEDGEMENTS. D.D.-J. and P.S.H. are equal first authors. We thank E. Harlow for discussions and reagents, and David Heimbach for peptides.

Paternal inheritance of mitochondrial DNA in mice

Ulf Gyllenstein*, Dan Wharton†, Agneta Josefsson* & Allan C. Wilson‡

* Department of Medical Genetics, Biomedical Center,

University of Uppsala, Box 589, S-751 23 Uppsala, Sweden

† New York Zoological Society, Bronx Zoo, New York 10460, and

Department of Biological Sciences, Fordham University, Bronx, New York 10458, USA

‡ Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720, USA

FOR nearly 20 years it has been assumed on the basis of low-resolution experiments that mitochondrial (mt)DNA, in contrast to the genes in the nucleus, has an exclusively maternal mode of inheritance in animals¹. Using the polymerase chain reaction^{2,3}, paternally inherited mtDNA molecules have now been detected in mice at a frequency of 10^{-4} , relative to the maternal contributions. These mice were hybrids between two inbred strains (C57BL/6J and *Mus spretus*) whose mtDNAs can be distinguished easily. This new mode of inheritance provides a mechanism for generating heteroplasmy and may explain mitochondrial disorders exhibiting biparental transmission.

Previous studies have shown that, in an average individual,

less than one mtDNA in a thousand is derived from the father¹. To perform a more sensitive test of paternal contribution we tried to build up a higher concentration of the paternal type by backcrossing of mice and made use of the polymerase chain reaction (PCR) to amplify paternally inherited mtDNA selectively. We crossed the mouse inbred strain C57BL/6J, hereafter referred to as C57BL, with a strain of *Mus spretus*. Hybridization between these yields fertile F1 females and sterile F1 males. A female hybrid from the cross ♀ *M. spretus* × ♂ C57BL was mated to a C57BL male to form the first generation of the C57BL backcross line. This process of repeatedly mating the female lineage stemming from *M. spretus* to males of the C57BL lineage was continued for 26 generations, to increase the amount of paternally inherited mtDNA to the point of detectability. Similarly, in a reciprocal cross, a female F1 hybrid obtained by mating a ♀ C57BL to a ♂ *M. spretus* was used to found a backcross lineage in which the female progeny bearing mtDNA of C57BL type were mated in nine generations to *M. spretus* males. Sublines were also created by mating backcross individuals among themselves, to determine whether paternally contributed mtDNA would persist in the strain.

A segment from base position 15,498 to 16,074 of the mitochondrial control region was chosen as a target for amplification (Fig. 1). The species-specific oligomers for this region will prime the synthesis of two fragments of 102 base pairs (bp) (B-G, A-H) and 577 bp (B-D, A-C), respectively (Figs 1 and 2a).

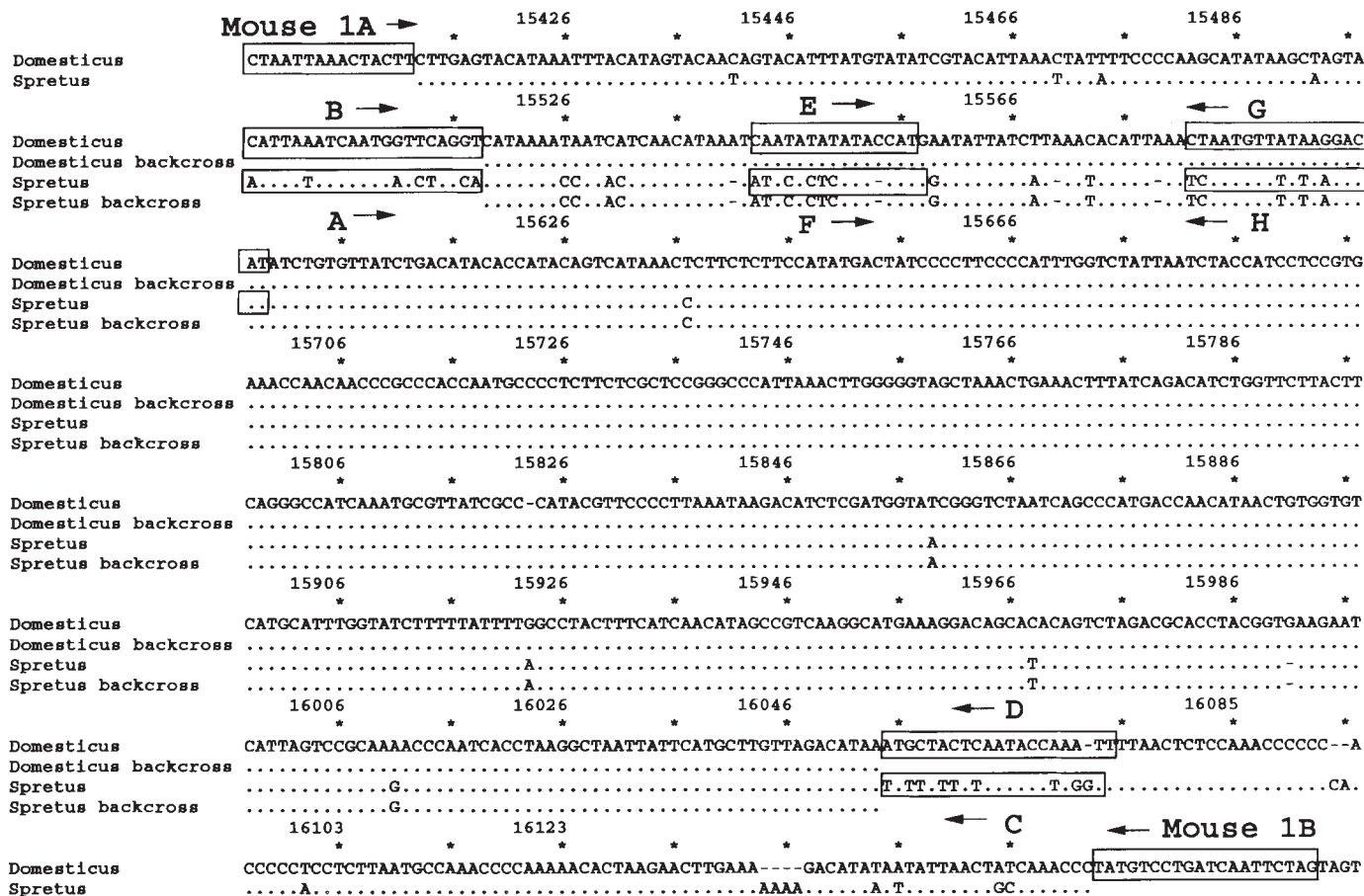


FIG. 1 Base sequence for the control region of the mitochondrial genome of C57BL, *M. spretus* and individuals from the backcross strains. Dots indicate identity with the C57BL sequence and dashes gaps. Locations of primers are indicated by boxes (A, 5'-AATTATATCAATGATCTAGCA-3'; B, 5'-CATTAATCAATGGTTCAGGT-3'; C, 5'-ACCTAGGTATTAACAAAAA-3'; D, 5'-AATTGGTATTGAGTAGCAT-3'; E, 5'-CAATATATATACCAT-3'; F, 5'-ATACATCATACATG-3'; G, 5'-ATGCTCTATAACATTAG-3'; H, 5'-ATGCTCTAAAA-

ACATTGA-3'). The C57BL sequence has been corrected relative to Bibb *et al.*¹⁷ at positions 15,823 and 16,119 (E. Prager, personal communication). The control region of C57BL and *M. spretus* was amplified using the primers Mouse 1A (15,393-15,412, 5'-AGAATTCGATTCTAATTAACACTT-3') and Mouse 1B (16,167-16,186, 5'-CTAGAATTGATCAGGACATA-3') and sequenced directly¹⁸.

Ten mice analysed from the strain that was backcrossed for 26 generations and two mice from the strain backcrossed for 23 generations to C57BL yielded PCR amplification products with the primer pairs diagnostic for paternally inherited mtDNA (B-D, Fig. 2a and Table 1; B-G, results not shown). These

products hybridized only with the C57BL oligonucleotide probe (E) (Fig. 2a) and sequence analysis showed the 577-bp amplified product from two backcross mice to be identical to the sequence of the C57BL parental strain (Fig. 1).

Likewise, all 14 mice examined from the strain backcrossed

TABLE 1 Paternally inherited mtDNA in backcross mice and lines with matings between backcross individuals

Type of backcross	Number of individuals	Kidney	Paternal mtDNA detected Liver	Heart	Proportion of paternal mtDNA
C57BL backcross*					
B23	2	+	+	ND	10^{-4}
B26	10	+	+	+	10^{-3}
<i>M. spretus</i> backcross*					
B8	12	+	ND	ND	10^{-4}
B9	2	+	+	ND	10^{-4}
C57BL backcross subline†					
B8s14	5	+	+	ND	10^{-3}
<i>M. spretus</i> backcross subline‡					
B9s2	3	+	+	+	10^{-4}

ND not determined; +, amplification product obtained.

* The first backcross generation (B) was generated by mating females from the interspecific cross (F1) to males of the parental species.

† The B8s14 subline was initiated with mice at the eighth generation of backcrossing and continued for 14 generations.

‡ The B9s2 subline was initiated with mice at the ninth generation of backcrossing and continued for two generations.

a *M. domesticus* primers B-D

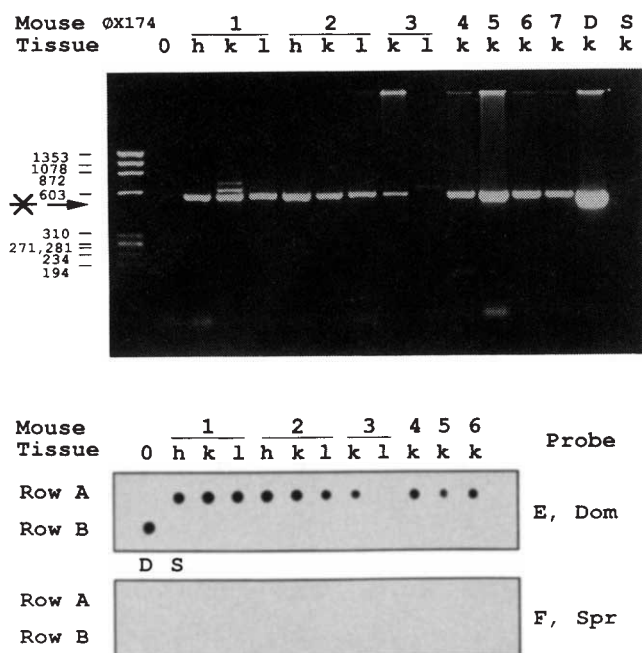
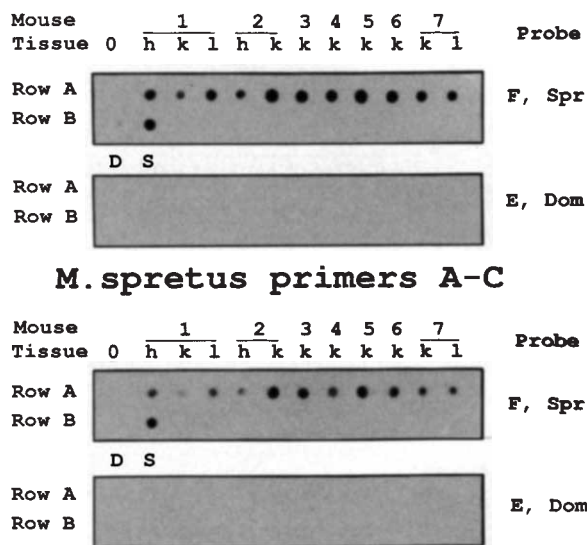


FIG. 2 a, Amplification of paternally inherited mitochondrial DNA in C57BL backcross mice using primers B and D. Upper panel, amplification products separated on a 2% agarose gel. Samples: 1 and 2, mice backcrossed to C57BL for 26 generations (B26); 3, mouse backcrossed to C57BL for 23 generations (B23); 4-7, mice generated by 14 generations of interbreeding among members of C57BL backcross strain (B8s14); l, liver; h, heart; k, kidney; 0, PCR performed without target DNA; D, C57BL parental strain; S, *M. spretus* parental strain; Φ X174, 0.5 μ g DNA from phage Φ X174 digested with *Hae*III. Lower panel, dotblot of the same amplification products above (excluding mouse 7). The parental strains D and S are shown in row B of each part of the lower panel. b, Amplification of paternally inherited mitochondrial DNA in *M. spretus* backcross mice using primers A and H and A and C. Upper panel, dotblot of products obtained with primers A and H. Samples: 1 and 2, mice generated by 2 generations of interbreeding among members of *M. spretus* backcross strain (B9s2); 3-6, 4 mice generated by 8 gener-

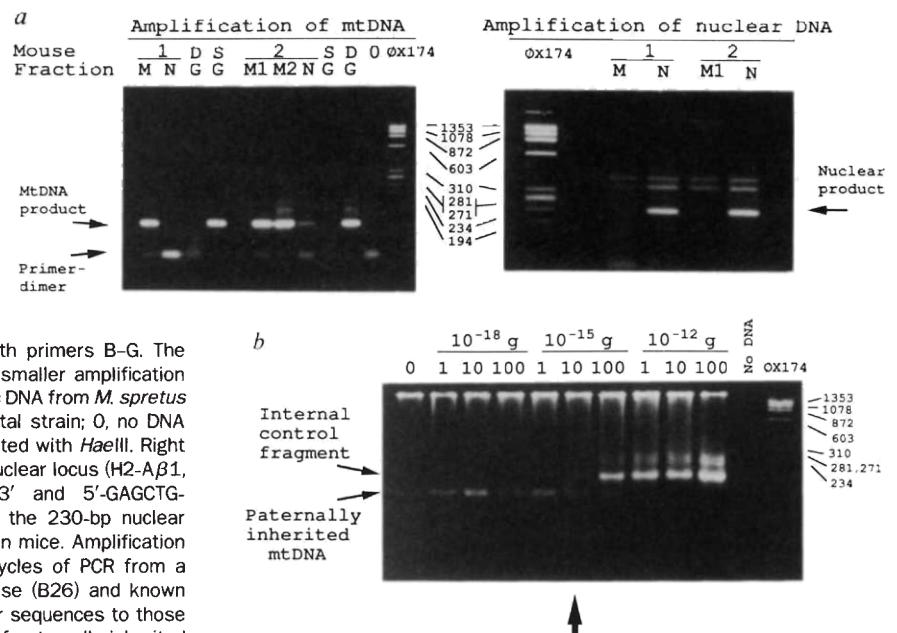
b *M. spretus* primers A-H



ations of backcrossing to *M. spretus*; 7, mouse generated by 9 generations of backcrossing to *M. spretus* (B9); l, liver; h, heart; k, kidney; 0, PCR performed without target DNA; D, C57BL parental strain; S, *M. spretus* parental strain. Lower panel, dotblot of amplification products obtained with primers A and C. The same individuals as in upper panel were analysed.

METHODS. The parental strain C57BL/6J was obtained from The Jackson Laboratory and contains mtDNA of *Mus domesticus* type^{10,18,19}. The Azrou (Morocco) strain of *Mus spretus* was obtained from R. Sage. For the isolation of mitochondria and nuclei, the tissues were homogenized in 0.25 M sucrose, 10 mM Tris-HCl, 10 mM EDTA, pH 7.5, and nuclei were removed by centrifugations at 1,000 g. The supernatant was spun at 10,000 g, the pellet resuspended in 0.25 M sucrose and the mitochondria isolated on a sucrose step gradient (0.25 M, 0.4 M, 0.5 M). Mitochondrial DNA was amplified by 25-45 cycles of PCR^{2,3} from 1 μ g total genomic DNA or 10 ng purified mtDNA, by incubation for 1 min at 94 °C followed by 30 s at 45 °C (primer pairs B-G and A-H) or 55 °C (primer pairs B-D and A-C) and 1 min at 72 °C in the presence of 2.5 U *Taq* polymerase (Perkin-Elmer Cetus) per 100 μ l and 0.5 μ M oligonucleotides (see Fig. 1). Amplified DNA (50 ng) was crosslinked by ultraviolet light to a nylon membrane (Genatrans, Plasco Inc.), hybridized with α -³²P-labelled oligonucleotides in 5 $\times$ SSPE, 0.5% SDS at 35 °C for 2 h, washed at 42 °C in 2 $\times$ SSPE, 0.1% SDS and exposed to Kodak X-AR film for 1-24 h (1 $\times$ SSPE=0.18 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 mM EDTA).

FIG. 3 *a*, Demonstration that the paternally inherited mtDNA does not represent a segment transferred to the nucleus. Left panel, DNA from purified mitochondria (M) and nuclei (N) from two backcross mice (mouse 1, *M. spretus* backcross mouse (B8); mouse 2, C57BL backcross mouse (B23)) was amplified for the paternally inherited mtDNA type. Two preparations of mitochondria (M1, M2) were obtained from mouse 2: M1 was incubated with DNase I for one hour prior to the lysis to remove nuclear DNA that was potentially carried on the outside of the mitochondrial membrane. The first four lanes from the left are amplifications performed with primers A-H, while the next five lanes are amplifications performed with primers B-G. The arrows point to the 102-bp mtDNA target while the smaller amplification product represents a dimer of the primers. SG, genomic DNA from *M. spretus* parental strain; DG, genomic DNA from C57BL parental strain; O, no DNA added; Φ X174, 0.5 μ g DNA from phage Φ X174 digested with *Hae*III. Right panel: DNA from the same two mice amplified for a nuclear locus (H2-A β 1, primers 5'-CTCGATCCGCATGTGCTACTTCACCAACG-3' and 5'-GAGCTG-CAGGTAGTTGTGTCTGCACAC-3'). The arrow indicates the 230-bp nuclear target.



contained the equivalent of 10 fg internal control fragment of paternally inherited mtDNA in 1 μ g total genomic DNA.

to *M. spretus* yielded an amplification product with the primers diagnostic for the paternally inherited mtDNA (A-C and A-H). These products were shown to contain exclusively *M. spretus* mtDNA by oligonucleotide hybridization (Fig. 2b) and the nucleotide sequence of the amplification product obtained with primers A-C from two backcross mice was found to be identical to the sequence of the *M. spretus* parental strain (Fig. 1).

The five individuals examined from the line of mice created by mating C57BL backcross mice among themselves for 14 generations contained paternally inherited mtDNA molecules and the line with matings among *M. spretus* backcross individuals contained *M. spretus* mtDNA (Fig. 2a, b; Table 1). Thus, the heteroplasmic state has persisted for 2-14 generations on both *M. spretus* and C57BL nuclear backgrounds.

Paternally inherited mtDNA was amplified from DNA of purified mitochondria, whereas little or no amplification product was found in the nuclear fractions (Fig. 3a). The lack of mtDNA amplification products in the nuclear fractions shows that the mtDNA PCR products in the backcross lines must be of mitochondrial origin and not represent a mtDNA copy that has been incorporated into the nuclear DNA⁴.

The proportion of paternally inherited mtDNA molecules was estimated using a DNA segment with the same primer sequences as the mtDNA target as an internal control. This was added in known amounts to a series of reactions and amplified together with the true mtDNA target⁵. The equivalent of 1 fg of internal control segment of paternally inherited mtDNA was detected in the *M. spretus* backcrosses, corresponding to a proportion of paternally inherited mtDNA of 0.0001 in 1 μ g of total genomic DNA. The C57BL backcross lines were found to contain the equivalent of 10 fg of paternally inherited mtDNA, corresponding to a proportion of 0.001, in 1 μ g of total genomic DNA (Fig. 3b; Table 1). The proportion of paternal mtDNA that can enter the egg cytoplasm and persist is thus $1 \times 10^{-3}/26 = 4 \times 10^{-5}$ per generation for the C57BL backcross, and $1 \times 10^{-4}/9 = 1.1 \times 10^{-5}$ for the *M. spretus* backcross. Because the midpiece of the sperm contains about 50 mtDNA molecules^{6,7}, a few of these may be transferred in each generation.

Paternal inheritance of mtDNA in interspecific crosses does not prove the occurrence of such a contribution intraspecifically. In *Drosophila*⁸ and *Mytilus*⁹, there have been indications of

incomplete maternal transmission. The mtDNA from the two mouse strains, because of the large genetic differences¹⁰, may escape mechanisms that inactivate paternal mitochondria coming from the same species and therefore are only slightly different. The nuclear background in backcross individuals may also select for the paternal mtDNA. A biparental inheritance of mtDNA provides a mechanism for generating mtDNA diversity within an individual^{9,11-14}. Although the absolute leakage of paternal molecules appears to be small, they could increase in frequency through bottlenecks during oogenesis to a dominant position¹⁵.

Paternal inheritance of mtDNA also means that mtDNA phylogenies are not exclusively matriarchal. Consequently, the effective population size for the species will be larger than for a strictly maternally inherited genome. In humans, estimates of the most recent common ancestor of all contemporary maternal lineages could thus be younger than a previous estimate of 200,000 years old, which was based on mtDNA comparisons¹⁶. □

Received 4 October 1990; accepted 21 May 1991.

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ACKNOWLEDGEMENTS. We are grateful for comments on the manuscript from A. Di Rienzo, U. Petterson, E. Prager, S. Pääbo, M. Stoneking, W. K. Thomas and L. Vigilant. This work was supported by a Knut and Alice Wallenberg Foundation Fellowship (U.G.), the Swedish Natural Science Research Council (U.G.) and the NSF (A.C.W.).

Anticodon and acceptor stem nucleotides in tRNA^{Gln} are major recognition elements for *E. coli* glutamyl-tRNA synthetase

Martina Jahn, M. John Rogers & Dieter Söll*

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511, USA

THE correct attachment of amino acids to their corresponding (cognate) transfer RNA catalysed by aminoacyl-tRNA synthetases is a key factor in ensuring the fidelity of protein biosynthesis. Previous studies have demonstrated that the interaction of *Escherichia coli* tRNA^{Gln} with glutamyl-tRNA synthetase (GlnRS) provides an excellent system¹ to study this highly specific recognition process, also referred to as 'tRNA identity'². Accurate acylation of tRNA depends mainly on two principles: a set of nucleotides in the tRNA molecule (identity elements) responsible for proper discrimination by aminoacyl-tRNA synthetases¹⁻³ and competition between different synthetases for tRNAs⁴⁻⁶. Elements of glutamine identity are located in the anticodon^{2,7-9} and in the acceptor stem region, including the discriminator base^{5,10-13}. We report here the production of more than 20 tRNA^{Gln} mutants at positions likely to be involved in tRNA discrimination by the enzyme. Unmodified tRNA, containing the wild-type anticodon and U or G at its 5'-terminus, can be aminocylated by GlnRS with similar kinetic parameters to native tRNA^{Gln}. By *in vitro* aminoacylation the mutant tRNAs showed decreases of up to 3×10^5 -fold in the specificity constant (k_{cat}/K_M)¹⁴ with the major contribution of k_{cat} . Despite these large changes, some of these mutant tRNAs are efficient amber suppressors *in vivo*. Our results show that strong elements for glutamine identity reside in the anticodon region and in positions 2 and 3 of the acceptor stem, and that the contribution of different identity elements to the overall discrimination varies significantly. We discuss our data in the light of the crystal structure of the GlnRS:tRNA^{Gln} complex^{15,16}.

To evaluate the contribution of the elements of glutamine identity in the anticodon and stem region of tRNA^{Gln}, we constructed two sets of mutant tRNA which were tested either *in vitro* for their aminoacylation with pure GlnRS or *in vivo* for their ability to suppress an amber codon in the *lacZ* gene¹⁷. For *in vitro* aminoacylation experiments we used RNA species transcribed from mutant tRNA^{Gln}G1 genes with base changes in the acceptor stem and anticodon regions (Fig. 1). The tRNA^{Gln} with G in position 1 can be aminoacylated only a third less well than tRNA^{Gln} made *in vivo* (Table 1). Thus, as is the case with some other tRNAs¹⁸⁻²⁶, the nine nucleotide modifications found in tRNA^{Gln} are not essential for aminoacylation although they enhance the reaction rate, possibly by ensuring a more tightly ordered tRNA structure^{26,27}. The results (Table 1) demonstrate that the K_M values vary 60-fold and k_{cat} values vary 2,700-fold between the best and the worst tRNA substrate. Therefore, the effect of the tRNA mutations is less on substrate binding than on catalysis.

In the crystal structure of the tRNA^{Gln}:GlnRS complex⁴, the first base pair (U1-A72) of tRNA^{Gln} is disrupted when complexed with the enzyme. Thus, loss of this base pair or deletion of G1 leaves aminoacylation virtually unaffected. But strengthening the base pairing makes tRNA^{Gln}G1-C72 an inferior substrate, an analogous situation to that of mutations of tRNA^{Met} toward glutamine acceptance²⁸. The discriminator base G73 is important for tRNA^{Gln} identity^{11,12} and helps maintain the hairpin structure of the 3'-end¹⁵. The tRNA^{Gln}G1A73 is

TABLE 1 Glutaminylation of mutant *E. coli* tRNA^{Gln} species by wild-type GlnRS

tRNAs	K_M (μM)	k_{cat} (s^{-1})	Relative k_{cat}/K_M
Gln (<i>in vitro</i>)			
G1	0.66	0.92	1.0
-C72	1.3	0.17	0.1
-U72	1.1	0.55	0.35
-A73	0.2	0.14	0.5
-C73	7.0	3.0×10^{-2}	3.0×10^{-3}
-U73	8.0	6.8×10^{-3}	6.0×10^{-4}
-U71	4.5	2.3×10^{-2}	2.0×10^{-3}
-A2-U71	10	1.1×10^{-2}	7.9×10^{-4}
-A2	8.0	0.12	1.3×10^{-2}
-U70	2.2	1.5×10^{-2}	4.9×10^{-3}
-A3-U70	3.3	4.6×10^{-2}	9.9×10^{-3}
-A3	3.3	2.3×10^{-2}	4.9×10^{-3}
-G38	3.0	6.0×10^{-2}	1.4×10^{-2}
-U37	1.0	9.3×10^{-3}	6.6×10^{-3}
-U36	0.5	1.0×10^{-2}	1.5×10^{-2}
-A36	6.0	5.0×10^{-3}	5.9×10^{-4}
-C35	6.7	3.4×10^{-4}	3.6×10^{-5}
-A35	5.0	3.7×10^{-3}	5.3×10^{-4}
-A34	2.5	6.5×10^{-4}	1.9×10^{-4}
$\Delta\text{G1-U72}$	0.83	0.6	0.5
U1*	0.15	0.2	0.95
U1-A36	6.6	3.6×10^{-3}	3.9×10^{-4}
Gln (<i>in vivo</i>)†	0.52	2.6	3.6

E. coli GlnRS and wild-type *E. coli* tRNA^{Gln} (anticodon CUG) were prepared as described previously³². Unmodified tRNAs with a 5'-triphosphate end were synthesized from mutant tRNA^{Gln} genes by transcription with T7 RNA polymerase. We constructed a tRNA^{Gln} gene with G in position 1 (from six oligonucleotides including the T7 RNA polymerase promoter), as native *E. coli* tRNA^{Gln} has a U in position 1 and as this nucleotide is not well suited for initiation of RNA synthesis with T7 RNA polymerase³³. The gene was ligated into a pUC119-derivative and transformed into *E. coli* DH5 α . Mutant tRNA genes were generated from this gene by oligonucleotide-directed mutagenesis. The mutation ΔG1 was a spontaneous deletion from the mutagenesis. The plasmid DNA was linearized with *Bst*NI and run-off transcripts prepared as described earlier¹⁰ by incubation at 37 °C for 1 h in a buffer containing 40 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 40 mM KCl, 5 mM DTT, 2 mM spermidine, ribonucleoside triphosphates (5 mM each), template DNA (0.2 mg ml⁻¹) and pure T7 RNA polymerase (25 μg ml⁻¹). As RNA transcripts were sometimes heterogeneous at their 3'-ends we used FPLC chromatography (Pharmacia) on MonoQ³⁴ for purification. In all cases the RNA from the MonoQ fraction appeared homogeneous after electrophoresis on denaturing polyacrylamide gels and had the correct CCA_{OH} end as determined by 3'-terminal nucleoside analysis of [³²P]pCp-labelled RNA (checked for most of the RNAs). The unmodified tRNAs containing U in position 1 (made by M1 RNA cleavage of a synthetic precursor tRNA) were purified by electrophoresis on 15% polyacrylamide denaturing gels. Aminoacylation was done at 37 °C in a buffer containing 30 mM Hepes (pH 7.5), 15 mM MgCl₂, 25 mM KCl, 5 mM DTT, 2 mM ATP, 0.1 mM [¹⁴C]-glutamine (237 mCi mmol⁻¹), with various concentrations of tRNA transcripts and GlnRS. *In vivo* isolated tRNA^{Gln} and tRNA^{Gln}G1 made *in vitro* were glutaminylated to 1,450 and 1,200 pmol/A₂₆₀, respectively. Aminoacylation conditions (for example ionic strength) affected the kinetic parameters of the transcripts slightly and the conditions used were optimal for the wild-type tRNA. Kinetic parameters for glutaminylation of the tRNAs were obtained by determining the initial rate of aminoacylation (over the first 2 min) and calculated using Lineweaver-Burke plots. The concentrations ranges for tRNA and glutamyl-tRNA synthetase were: for good substrates (0.03–1.0 μM RNA, 10 nM GlnRS), for poor substrates (0.1–5.0 μM RNA, 3 μM GlnRS). The 5'-triphosphate end did not affect aminoacylation as shown in control experiments.

* Unmodified tRNA^{Gln} of wild-type sequence; † tRNA^{Gln} of wild-type sequence made *in vivo*.

hardly impaired in aminoacylation, whereas tRNAs with a pyrimidine in this position show a decrease of k_{cat}/K_M by 300- and 1,700-fold, respectively. The G $\rightarrow$ A73 change may not cause a significant disturbance in the hairpin structure whereas a pyrimidine in position 73 would disturb the stacking with C75, which in turn might change the position of A76 in the active

* To whom correspondence should be addressed.

site of GlnRS. The bases G2 and G3 form specific hydrogen-bond interactions with GlnRS through a water molecule contributing to tRNA discrimination^{13,15}. Therefore we effected G → A changes in these positions which give rise either to mispairing or to double mutants with restored base pairing. In addition, single mutations created G-U pairs in these positions. With the exception of the A2 mutant, these tRNAs have reduced acylation rates. It seems that in addition to contacts made through the 2-amino group of G2 and G3 the exact positioning of these groups in the protein:RNA complex is important, as the helical irregularity introduced by the G-U pair leads to a large decrease in k_{cat} in tRNA^{Gln}U70 and tRNA^{Gln}U71. In all these cases the effects of the base changes may not be localized and may affect the RNA structure in a more global sense. It could also be argued from the data of the tRNA^{Gln}A2-U71 that a strong base pair is needed between positions 2 and 71 as this is the first base pair in the tRNA^{Gln}:GlnRS complex¹⁵.

The most severe impairment of GlnRS catalysis is seen with tRNAs mutated in the anticodon region. Mutations were made in each anticodon position and in positions 37 and 38, adjacent to the 3'-end of the anticodon. The latter position was chosen as earlier NMR studies²⁹ and the crystal structure¹⁶ indicated that GlnRS may affect the environment of pseudouridine. The much reduced k_{cat}/K_M value may be explained by the inability of tRNA^{Gln}G38 to form the unusual base pair in the complex¹⁶. Position 37 in tRNA often contains a modified purine, and a pyrimidine in this position (tRNA^{Gln}U37) is not a good substrate for GlnRS. This is to be expected as the nucleotide in position 37 is involved in pairing with U33 (ref. 16). With the exception of substitution of U in position 36 the other single base substitutions in the anticodon had large effects of k_{cat}/K_M . The large effect of the anticodon bases on tRNA identity is well illustrated by the detailed interaction of each anticodon base with GlnRS¹⁶. Large k_{cat}/K_M changes as a consequence of single base mutations in the anticodon have also been seen in the 'identity switch' experiments with tRNA^{Met}_f and tRNA^{Met}_m^{Met7,21,22,25}. Because the tRNA^{Gln} species with anticodon mutations are poor substrates for GlnRS they may be mischarged by noncognate aminoacyl-tRNA synthetases.

TABLE 2 Suppressor efficiency of *supE* mutants in *E. coli lacZ*₁₀₀₀

Mutants	β -Galactosidase activity (U)	Suppressor efficiency (%)
<i>su</i> -	0.8	0.5
<i>supE</i>	145.0 ± 6.0	100
G ₁ -C ₇₂	42.5 ± 1.3	29
A ₃ -U ₇₀	18.1 ± 0.4	12.5
U ₇₀	19.9 ± 0.8	13.7
C ₇₃	159.8 ± 9.5	110
U ₇₃	144.5 ± 5.2	100
A ₇₃	114.3 ± 2.0	78.8

The *supE* tRNA^{Gln} gene (anticodon CUA) was constructed in plasmid pGFI³⁵ from six synthetic oligodeoxynucleotides to give an *Eco*RI-*Bam*HI fragment. Mutants of the *supE* gene were made by oligonucleotide site-directed mutagenesis. For determination of the suppression of *lacZ*₁₀₀₀, the mutants were transformed in *E. coli* strain RS111 (F⁻, *lacZ*₁₀₀₀am^r, *trp*_{am}^r, *cys*_{am235}^r, *met*_{am3}^r, *rif*^r) followed by determination of β -galactosidase activity¹⁷.

Although a single nucleotide change (G → A36) makes tRNA^{Gln} a very poor substrate *in vitro* (Table 1), *in vivo* this tRNA^{Gln} is the functional *E. coli* amber suppressor *supE*. Thus, the suppressor assay is extremely sensitive³⁰. As suppression of amber codons is the basis for the *in vivo* assay, mutational changes in the anticodon cannot be assessed in this way. Therefore we constructed a set of mutants with changes in the acceptor stem region. The mutant suppressor tRNAs were tested in suppression of the glutamine-specific *lacZ*₁₀₀₀ amber mutation¹⁷ (Table 2). Mutations in the 3-70 base pair had a major effect on suppressor efficiency, in agreement with the results of the aminoacylation studies (Table 1). The mutant *supE*G1-C72 shows reduced suppression as expected from the *in vitro* aminoacylation (Table 1) and structural data¹⁵. But, this tRNA still acts as a suppressor *in vivo*. The mutations in the discriminator base to a pyrimidine had little effect on suppression *in vivo* (Table 2). This may imply that the considerable effect

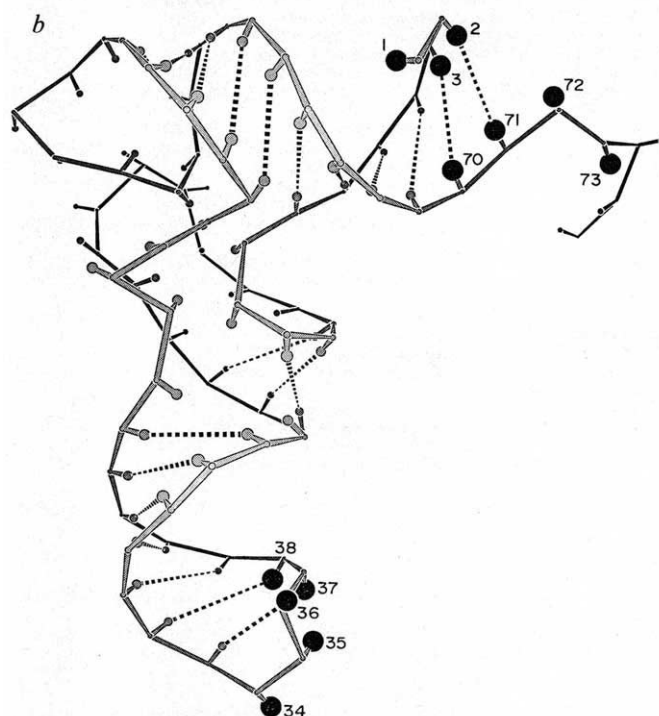
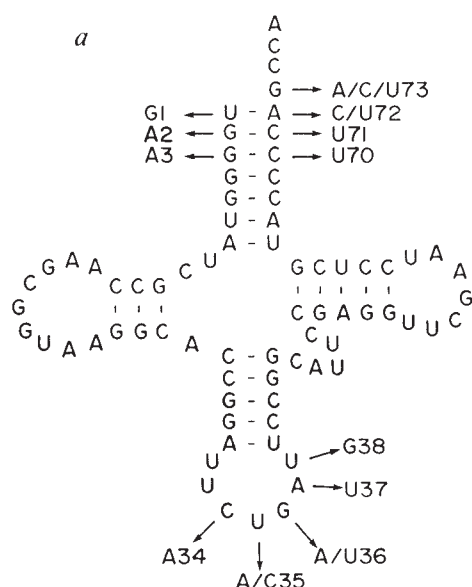


FIG. 1 *a*, The secondary structure of the unmodified tRNA^{Gln}₂ shown in cloverleaf form. The numbered nucleotides indicate the mutations made for transcription *in vitro* and expression *in vivo*, as described in the legends to Tables 1 and 2. *b*, The tertiary structure of the phosphate backbone (with bases shown as spheres) of *E. coli* tRNA^{Gln}₂ complexed with GlnRS with positions of mutated bases (shown in *a*) highlighted (●).

(Table 1) on *in vitro* aminoacylation of tRNA^{Gln}C73 and tRNA^{Gln}U73 is due to conformational change of the tRNA transcript. The mutation A73 has a small effect *in vivo* and may mimic the situation *in vitro*.

How does tRNA identify function? There are nucleotides at certain positions which make direct and specific interactions with parts of the protein. Such bases include positions 2, 3, 34–38, 70, 71 and 73 of the mutants tested^{15,16}. Other positions may also be important where bases do not make actual contacts with the enzyme but facilitate the maintenance of the correct RNA structure for specific interaction with the protein. But tRNA discrimination also involves negative elements that block recognition by noncognate aminoacyl-tRNA synthetases. These will only be seen if the positive tRNA^{Gln} identity elements are translated into a 'generic' tRNA context to determine glutaminylation efficiency.

Is there a threshold value for tRNA discrimination? Our data show that individual identity elements contribute variable amounts to the discrimination process (giving an overall discrimination value) and there is an indication that the contributions of the various elements are additive. The total discrimination value may be 'overdiscrimination' for *in vitro* aminoacylation, as several tRNAs which do not share all these identity elements can be charged *in vitro* with glutamine³¹. But a high discrimination threshold value is probably required to obtain the high specificity seen *in vivo*. Given that there are tRNA 'families' with related identity, within which single base changes may lead to identity switches, the recognition of certain nucleotides in tRNA may be shared by more than one enzyme. Therefore, competition between synthetases in the cell may ultimately determine the accuracy of *in vivo* aminoacylation of tRNA. □

Received 8 April; accepted 7 June 1991.

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ACKNOWLEDGEMENTS. We thank U. Burkard for help at the start of this project and M. Rould and T. Steitz for help and discussions. This work was supported by the NIH.

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Radioactive cell membrane labelling

Sue E. Slezak and Katharine A. Muirhead

Building upon earlier studies with fluorescent probes, the authors describe a new cell tracking compound, PKH95, with a radioactive signal, which has been developed specifically for high-sensitivity cell tracking and biodistribution studies.

METHODS to monitor cell trafficking and cell-cell interactions have become paramount to uncovering the mechanisms involved in the pathogenesis of diseases such as metastatic cancer, atherosclerosis, rheumatoid arthritis and others. A significant limitation to such studies has been the availability of probes that can be used for relatively long-term cell tracking. Ensuring that the probes do not perturb cellular functions and that they remain stably associated with the cells of interest is critical if a true picture of the disease process is to be obtained.

Although markers such as fluorescein and rhodamine isothiocyanate and the Hoechst dyes have been used for cell tracking, they have been limited to relatively short-term tracking studies (3–5 days). The problems encountered in applying these probes to longer term studies include leakage, transfer of label, toxicity or altered cell function caused either by the labelling process or by the probe itself. Similar problems have arisen in applying radioisotopes such as chromium-51, indium-111 or technetium-99m to the quantitative analysis of cell tracking and biodistribution. These problems typically result from chemical toxicity of the compounds used to load the isotope into the cell or from leakage due to lack of stable binding of the isotope to intracellular constituents¹.

Zyn-Linker technology was developed to improve cell tracking capabilities and permit a better understanding of various disease processes, the eventual goal being to use this information to design more effective therapeutic agents. The fluorescent compounds PKH2 and PKH26 (ref. 2) solve many of the stability and toxicity problems associated with traditional cell tracking agents and have proved useful for tracking circulating cells and for visual observation of cell localization in tissues. They have also been used for tracking cells over periods of weeks to months^{3,5} and have been found to have no effect on a variety of cell functions^{6,7}. While these compounds permit visual observation of cell localization, a major limitation of all fluorescent tracking probes is the difficulty in quantitatively determining the number of cells migrating to a particular site.

Radio-tracking capabilities

By expanding upon the original fluorescent technology, an iodine-125 Zyn-

Linker (¹²⁵I-PKH95) has been developed that permits radioactive cell tracking and accountability. This probe is designed to be used alone for biodistribution studies or in combination with the fluorescent probes to allow visual observation of cell localization. Unlike many other radioisotopes used for cell tracking, PKH95 partitions into the lipid regions of the cell membrane rather than the cytoplasm, providing enhanced retention due to its aliphatic nature. This mechanism of incorporation allows the probe to be used to label prokaryotic cells and eukaryotic cells, as well as viruses, liposomes and other membrane-containing particles.

PKH95 exhibits very good membrane binding stability, with a half life for loss of label from red cells in *in vivo* circulation of greater than 40 days. The chemical design of the molecule by Brian Gray and Kamlesh Sheth was structured to maximise stability of the radioactive isotope and prevent the problem of deiodination of the molecule *in vivo*. Less than 0.1% of injected counts have been found in the thyroid of animals injected with labelled red cells or lymphocytes and followed for one to six weeks⁸.

The number of PKH95 molecules that can be incorporated per cell without detrimental effects on cell viability or proliferation has proved somewhat lower than comparable values for PKH26, due to the chemical bulk of the iodine atom. The values obtained are in the order of 10⁵ molecules per red cell, 5 × 10⁶ per cell for YAC-1 cultured lymphoma cells, and 10⁷ per cell for murine tumour infiltrating lymphocytes (TILs) or lymphokine activated killer (LAK) cells⁸. At specific activities of 25–50 mCi μmol⁻¹, these values translate to approximately 2 × 10⁻⁹ μCi per cell (≈0.003 c.p.m. per cell) for red cells, 10⁻⁷ μCi per cell (≈0.15 c.p.m. per cell) for YAC-1 cells and 2 × 10⁻⁸ μCi per cell (≈0.3 c.p.m. per cell) for TILs or LAK cells, values that are more than adequate for most *in vivo* cell tracking purposes but which are not radiotoxic. Microdosimetry studies indicate that at least 10,000 disintegrations are required for membrane-bound ¹²⁵I to cause any significant effect; a cell labelled with 5 × 10⁻⁸ μCi would require 58 days to receive this level of exposure⁹. The efficiency of ¹²⁵I-PKH95 incorporation can vary from 10–90% and

depends on the concentration of both probe and cells in the labelling step, and on the particular cell type. Use of high probe concentrations and/or low cell concentrations results in a lower overall incorporation efficiency but maximal counts per cell.

In vivo tracking

John McAfee and colleagues at George Washington University have characterized the labelling of neutrophils with ¹²⁵I-PKH95, prefatory to studies in various inflammation models. They found that the cell labelling yield ranged from 64–74% and was not affected by prior cell labelling with the standard leukocyte labelling agent, ¹¹¹In-oxine. They also found that labelling was highly stable to serial washing of the labelled cell suspensions in plasma or saline, and that harvesting and labelling procedures did not induce any apparent morphological changes (J. McAfee, personal communication).

Studies of LAK cells and TILs, carried out in collaboration with James Yang of the National Cancer Institute, indicate that proliferation rate and *in vitro* tumour cytotoxicity are not altered by labelling with ¹²⁵I-PKH95 (ref. 10). The *in vivo* therapeutic efficacy of LAK cells and TILs against lung micrometastases is also unaffected by PKH95 labelling (see Fig. 1). Yang plans to conduct further studies on the natural trafficking pattern

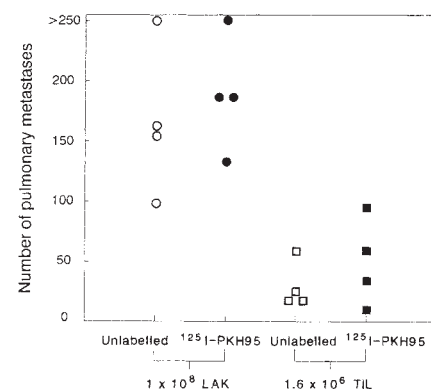


FIG. 1 Therapeutic efficacy of ¹²⁵I-PKH95 labelled or unlabelled murine lymphokine activated killer (LAK) cells or tumour infiltrating lymphocytes (TILs) against lung metastases. Cellular immunotherapy significantly reduced the number of lung metastases compared with animals treated with recombinant IL-2 only (>250 metastases per lung); the efficacy of labelled and unlabelled cells did not differ significantly.

of TILs to various sites of metastatic tumour and of manipulations that alter trafficking patterns, in the hope of optimizing the efficacy of immunotherapy (J.C. Yang, personal communication).

Once appropriate labelling levels for a given cell type are established, a variety of methods can be used for *in vivo* cell tracking. The distribution of ^{125}I -PKH95-labelled cells can be followed at the whole body level by determining the fraction of injected counts recovered in specific organs of interest (see Fig. 2). In small animal models, whole body gamma imaging can also be used, although the

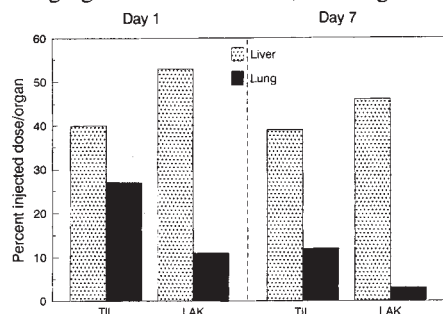


FIG. 2 Biodistribution of ^{125}I -PKH95-labelled murine TILs or LAK cells in normal mice determined by gamma counting of organs at one and seven days post-injection.

relatively low energy gamma particle emitted by ^{125}I gives poorer resolution than radionuclides used in clinical practice, such as $^{99\text{m}}\text{Tc}$ or ^{111}In . It is also possible to visualize the microdistribution of labelled cells within an organ in several ways. For detection of ^{125}I -PKH95-labelled cells in tissue sections, autoradiographic methods may be used¹¹. Alternatively, cells may be co-labelled with ^{125}I -PKH95 and PKH26, and PKH26 fluorescence can be used to characterize the localization of labelled cells. Frozen sections are recommended when carrying out histological studies, as organic solvents may remove the lipophilic linkers.

Future prospects

Zyn-Linkers bearing more optimal gamma imaging atoms than ^{125}I -PKH95 are under development so that sequential studies in a single animal, particularly in large, expensive and/or scarce animal models, can be carried out. In this context, a model $^{99\text{m}}\text{Tc}$ -Zyn-Linker has been synthesized and is being tested in biological systems.

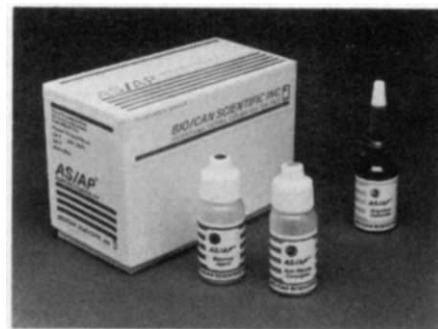
A Zyn-Linker derivative has also been developed that allows conjugation of Zyn-Linkers to a variety of active moieties in addition to chelators for radionuclides. For instance, Zyn-Linker-conjugated biotin has been demonstrated to retain its avidin-biotin binding capabilities. Substance P, an eleven amino acid vasoactive peptide, has been conjugated and shown to retain *in vivo* activity¹².

Labelling and detection

This week's labelling technology product line-up includes reagents for three-colour cell analysis kits, for end-labelling oligos and an ethidium bromide stain substitute.

THE immunocytometry division of Becton Dickinson has introduced three directly conjugated peridinin chlorophyll protein reagents (PerCP) for **three-colour cell analysis** on a single-laser flow cytometer (*Reader Service No. 101*). PerCP single dye conjugate reagents, which are designed for use with the company's Simultest reagents (fluorescein isothiocyanate and phycoerythrin) and the FACScan single-laser flow cytometer, provide simultaneous detection of single-, double- and triple-stained cells through a one-step staining process. The reagents have a peak emission spectrum of 677 nm, which minimises the spectral overlap with FITC and PE dyes, Becton Dickinson says. Stated applications include the study of activation, differentiation and proliferation of T-lymphocytes, which may be useful in AIDS and autoimmune disease research, and in the characterization of T-cell lymphomas and leukaemias. The PerCP reagents, Anti-Leu-4 (CD3) and Anti-Leu-2a (CD8) each cost \$550 (US), which is sufficient for 100 tests; Mouse IgG1G₁ costs \$400 (US) for 100 tests.

High sensitivity and low background staining are features of the AS/AP (amplified substrate/alkaline phosphatase) Immunostaining Kits, says Accurate Chemical and Scientific (*Reader Service No. 102*). The **immunostaining kits** use an amplified substrate system and an alkaline phosphatase-conjugated second antibody. Accurate claims that the system is up to ten times more sensitive than either the PAP or ABC staining methods, and so should provide a more economical use of primary antibodies. Two kits are available: one for the



Two-step immunostaining — see Accurate.

detection of all rabbit polyclonal primary antibodies, and the second for mouse monoclonal primary antibodies. All three reagents — blocking agent, conjugate and chromogenic substrate — are supplied in convenient dropper bottles as ready-to-use solutions. Both immunostaining kits can be used with paraffin-embedded or frozen tissue sections. And, both are available in two sizes with sufficient reagents for processing 75 and 300 slides, costing \$85 (US) and \$270 (US), respectively.

The Lumi-Phos substrate kit from Cambridge Research Biochemicals can be used for the **detection of alkaline phosphatase-conjugated molecules**, such as antibodies, oligonucleotides or double-stranded DNA probes (*Reader Service No. 103*). Two sizes of kit are available, containing blocking buffer and either 35 ml or 100 ml of the Lumi-Phos 530 substrate. The Lumi-Phos substrate is supplied as a ready-to-use spray formulation consisting of Lumigen PPD, buffer, CTAB and enhancer. Enzymatic removal of the phosphate group from the stable dioxetone produces an un-

We hope, initially, to utilize the stable membrane binding characteristics of Zyn-Linkers in products that enhance selective retention of drugs or other bioactive molecules at known disease sites and, eventually, to harness the power of cell trafficking to deliver Zyn-linked therapeutic agents to occult disease sites. □

Sue E. Slezak and Katharine A. Muirhead are at Zynaxis Cell Science, 371 Phoenixville Pike, Malvern, Pennsylvania 19355, USA. For more information, fill in reader service number 100.

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stable intermediate, which then decomposes to provide the observed chemiluminescence, CRB says. The enhancer system consists of a fluorescein-derivatized co-surfactant in miscelles, which, the company says, provides a 370-fold increase in chemiluminescent efficiency. The blocking buffer, supplied as a 10× concentrate, is based on casein and is optimized for use in Southern, northern, or western blotting applications using nylon or PVDF membranes. The chemiluminescent signal is recorded on X-ray film.

Stain selection

NucliStain, an alternative to ethidium bromide, is a **positive stain concentrate** from National Diagnostics that is designed for the rapid detection of double- and single-stranded DNA and RNA in agarose and polyacrylamide gels (*Reader Service No. 104*). Although NucliStain has a comparable sensitivity to ethidium bromide, both stains can detect as little as 50 ng of DNA, the real advantage of NucliStain, the company says, is that results are visible under normal lighting conditions. To use, the stain is simply diluted; separated DNA appears as dark blue bands on a light blue background.

For the rapid and sensitive **detection of proteins and nucleic acids**, Boehringer recommends its Silver Stain Kit (*Reader Service No. 105*). The company claims that the kit provides a detection sensitivity that is more than 100 times that of Coomassie Blue staining methods. In addition, the kits are function tested for their ability to detect at least 10 ng



Silver stain in kit form.

bovine serum albumin in one hour. The four-step procedure for using the silver stain is as follows: fix the gel, apply the sensitizer, stain the gel and then develop the stain to visualize the bands.

End labelling

Enzo Diagnostics has extended its non-radioactive labelling technology to the **biotin-labelling of synthetic oligomeric probes** (*Reader Service No. 106*). The method, which is based on the use of terminal deoxynucleotide transferase to catalyse the 3'-hydroxyl addition of biotin-11-dUTP, results in DNA probes with single-stranded biotinylated 'tails'.

Unlike nick translation, terminal labelling is template-independent and there is net DNA synthesis, Enzo says. The \$225 (US) Terminal Labelling Kit labels synthetic oligonucleotide probes with a biotin tail. But, before terminal labelling, the probes must first be purified, either by polyacrylamide gel electrophoresis or HPLC, to ensure that only complete sequences are labelled. According to Enzo, the length of the biotinylated tails is dependent upon both the nucleotide concentration and the time of reaction. The terminal-labelled probes can be used with standard hybridization procedures; the hybridized biotinylated probes can then be detected with an appropriate streptavidin-biotin detection complex such as DETEK *hrp* (horseradish peroxidase) or DETEK *alk* (alkaline phosphatase).

Tropix announces the availability of its new **non-radioactive 3' end-labelling kit**, which can be used to insert several biotin-labelled nucleotides onto the 3' terminus of a purified oligonucleotide (*Reader Service No. 107*). The entire procedure, from probe labelling and hybridization to chemiluminescent detection, takes less than five hours, Tropix says. The kit uses the enzyme Terminal deoxynucleotidyl Transferase (TdT) to incorporate biotin-11-dUTP and dATP in a concentration that has been optimized for assay sensitivity and specificity. Detection procedures are described for the chemiluminescent detection of the biotin-labelled oligonucleotides using the Tropix Southern-Light chemiluminescent detection system. Each \$125 (US) test kit also includes a 5× 'tailing' buffer and both labelled and unlabelled pBR322-35 mers as assay controls. Sufficient reagents are provided to perform up to 50 labelling reactions, each of 90 picomoles. The 3' end labelling kit when sold in conjunction with the company's end labelling chemiluminescent detection system costs \$325 (US).

Practical hybridization

Overnight hybridizations could be a thing of the past, according to Stratagene whose new QuikHyb **hybridization solution** reduces the time required for nucleic acid hybridization to membrane-immobilized target sequences from the typical 12 to 24 hours down to 1 to 2 hours (*Reader Service No. 108*). The hybridization solution produces a sub-picogram sensitivity with minimal background, Stratagene says. It is suitable for use in Southern, northern, plaque and colony hybridizations using both radioactive and non-radioactive probes, and has been used successfully with both nylon and nitrocellulose membranes. QuikHyb can be purchased separately in 250-ml quantities for \$250 (US) or in combina-

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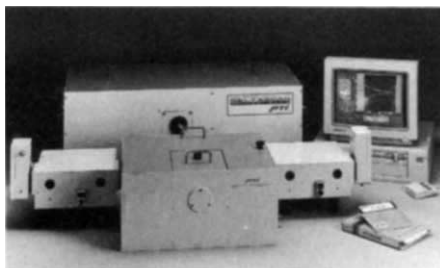
tion with the \$185 (US) Prime-It or \$185 (US) FLASH Prime-It random primer labelling systems (125 mls).

The *In-Situ* Instructor hybridization kit, developed in conjunction with Dr Howard Pringle from the Department of Pathology at Leicester University, is available from British Bio-technology (Reader Service No. 109). The kit, which can be used to demonstrate the detection of insulin mRNA in the β cells of rat pancreas, contains all the reagents for proteinase K pretreatment, RNase treatment, fixation, prehybridization, washes, and the detection of insulin mRNA in paraffin-embedded rat pancreas sections. Insulin mRNA is detected using a cocktail of six oligonucleotide probes, which are coupled to the hapten fluorescein isothiocyanate (FITC), and which are complementary in sequence to rat preproinsulin mRNA. Hybridized oligonucleotides are detected using a two-tier antibody system. The £150 (UK) *In Situ* Instructor training kit demonstrates the specific detection of target (preproinsulin) mRNA in a precise cellular location (pancreatic β cells) with retention of tissue morphology; the destruction of hybridization signal by RNase, indicating the target sequence is RNA; the importance of using a range of proteinase K concentrations for tissue pretreatment;

and the applicability of the technique to processed paraffin-embedded tissue.

Detection devices

The new Deltascan Model 4000 Series of high-speed, scanning dual-wavelength spectrofluorometers from Photon Technology International are designed for researchers studying calcium, pH and other intracellular ions, such as sodium and potassium, in living cells (Reader Service No. 110). The fluorescence systems provide a host of new features. In addition to the data analysis and presentation software, new hardware features include a bifurcated quartz fibre-optic bundle, improved monochromators, a new photometer with photon-counting detectors, and a new programmable shutter for reduced sample photodegradation, PTI says. For maximum flexibility, the Deltascan systems can be configured for use with an inverted epifluorescence microscope or a cuvette-based sample compartment. Single- and dual-emission versions are available. The Deltascan fluorescence



Fluorescent systems for calcium research.

systems range in price from \$48,000 to \$54,000 (US).

The high counting efficiency of multiple well-type detectors combined with the high throughput capability of advanced robotics and easy-to-use software are features of the new WIZARD gamma counter from Pharmacia (Reader Service No. 111). The counter, which has 10 detectors and a sample capacity of 550 or



High RIA throughput with the WIZARD.

1,000, has a throughput capacity of up to 500 samples per hour, Pharmacia says. In addition to processing single- and dual-labelled RIAs, the counter can be used with Chromium Release and Shilling tests. Operators can set the instrument to operate in automatic or manual mode. Samples in vials of all shapes and sizes up to 13 × 90 mm can be counted.

Mail order

A wealth of new products are featured in the latest edition of the Bioprobes catalogue from Molecular Probes (Reader Service No. 112). Three new Ca^{2+} indicators, Calcium Green, Calcium Orange and Calcium Crimson, have been developed that are excited by visible light. The three indicators respond to Ca^{2+} binding by increasing their fluorescence emission intensity with little wavelength shift, the company says. Fura Red, a longer wavelength version of fura-2, is also available and should provide new possibilities for the ratio imaging of Ca^{2+} (and simultaneously pH) in single cells in imaging and flow cytometry. SNARF calcein and SNAFL calcein are dual-emission pH indicators that are suitable for use with a single argon laser in applications such as confocal microscopy, flow cytometry and ratio imaging. The catalogue also lists coloured and fluorescent latex microspheres, fluorescent secondary antibodies, an intracellular glutathione indicator and new BODIPY lipid probes.

Seventy five new products that focus on non-radioactive detection for immunohistochemical, enzyme immunoassay and transfer blot techniques are featured in the 1991 catalogue from Vector Laboratories (Reader Service No. 113). Key products include Vectabond Reagent, a new tissue section adhesive, and Neurobiotin, an intracellular label for neurons. Also listed are peroxidase and alkaline phosphatase substrate kits and biotinylation reagents, including a water-soluble derivative for protein labelling and a sulphhydryl reactive label. Vector also offers high-purity Agarose Protein A for use in immunoglobulin purification, such as the isolation of IgG from mouse ascites fluid, immunoprecipitation of antigens, and other applications involving specific immunoglobulin binding. The Protein A, which is attached through a stable linkage to 4% agarose beads, has a typical binding capacity of >10 mg human IgG per ml of gel. □

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